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1. Wang L, et al. Int J Oncol. 1999 Apr;14(4):695-701.

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Bone marrow-derived dendritic cells incorporate and process hydrophobized polysaccharide/oncoprotein complex as antigen presenting cells

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Abstract. We have previously shown that a novel hydrophobized polysaccharide/oncoprotein complex vaccine can induce immune responses against the HER2/*neu*/c-*erb*B2 (HER2) expressing tumors. Bone marrow-derived dendritic cells (DCs), as antigen presenting cells (APCs), are the first candidates for presentation of tumor antigens. The aim of this study was to see whether DCs are able to elicit antigen specific host immune responses by stimulating the proliferation of T cells after exposure to cholesteryl group bearing pullulan (CHP) and HER2 protein complex. Vaccination by CHP-HER2 complex was as effective as cholesteryl group bearing mannan (CHM) and HER2 complex on which we reported previously. Immunization of mice with HER2 expressing CMS17HE tumor cells generated both $CD4^+$ T cells and $CD8^+$ T cells reactive with CHP-HER2 complex pretreated DCs. In addition, immunization with either CHP-HER2 complex or HER2 protein alone could also generate both $CD4^+$ T cells and $CD8^+$ T cells specifically reactive with CHP-HER2 complex pretreated DCs. The complete rejection of tumors occurred when immunization with CHP-HER2 complex pretreated DCs was started 10 days after tumor inoculation. Therefore, bone marrow-derived DCs pretreated with hydrophobized polysaccharide/oncoprotein complex are a powerful tool for enhancing the effectiveness of oncoprotein for anti-tumor vaccination, opening new options for immune cell therapy.

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Abbreviations: CHM, cholesteryl group bearing mannan; CHP, cholesteryl group bearing pullulan; CAB, carbonic anhydrase II; HER2, HER2/*neu*/c-*erb*B2; CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; rmGM-CSF, recombinant murine granulocyte/macrophage-colony stimulating factor; MHC, major histocompatibility complex; APCs, antigen presenting cells; MR, mannose receptor

Key words: dendritic cells, hydrophobized polysaccharides, HER2/neu/c-erbB2 oncoprotein, tumor vaccine

Introduction

Recently new advances allow the realization of potent vaccination schemes: characterization of tumor antigens (1-3), development of molecular delivery systems (4,5) and manipulation of antigen presenting cells (APCs), such as dendritic cells (DCs) (6). The proto-oncogene HER2/*neu*/c-*erb*B2 (HER2) is overexpressed in a variety of human cancers such as breast, ovarian, gastric and renal cancers as well as in other tumor entities (7-11). We have demonstrated that HER2 can be an effective target molecule for specific immune responses against $HER2^+$ tumor cells in a syngeneic murine system (4,12). We also reported recently a novel hydrophobized polysaccharide/HER2 oncoprotein complex vaccine, which can induce strong cellular and humoral immune responses against HER2 expressing tumor (4). A truncated protein consisting of the 147 N-terminal amino acids of the proto-oncogene HER2 was complexed with hydrophobized polysaccharides, cholesteryl group bearing mannan (CHM) and cholesteryl group bearing pullulan (CHP), to form nanoparticles. In mice immunized with these complexes, HER2 specific $CD8^+$ cytotoxic T lymphocytes (CTLs) could be generated and prevented growth of subsequently inoculated HER2 expressing tumors. The CTL

generated by the immunization with CHM-HER2 complex recognized a peptide spanning the positions 63 to 71 of HER2 (HER2 p63), a part of the truncated protein used for vaccination. $CD8^+$ T cells played a major role in the effector phase *in vivo* tumor rejection of host vaccinated with CHM-HER2 complexes. These observations strongly suggested that hydrophobized polysaccharide/truncated HER2 oncoprotein complex could be efficiently delivered to the pathway to produce target antigen peptides recognized by $CD8^+$ CTLs and their precursors in the context of MHC class I molecules. In addition, mice immunized with CHM-HER2 complexes could produce an extremely high titer of IgG antibodies against HER2 protein indicating a possible activation of helper $CD4^+$ T cells. Detailed mechanisms of antigen presentation in animals immunized by hydrophobized polysaccharides/oncoprotein complexes are still unknown. It has been reported that DCs, as professional APCs, could process and present antigen peptides to T cells efficiently, and could induce anti-tumor immunity (13-18).

We therefore questioned in this study whether bone marrow-derived DCs can incorporate CHP-HER2 complex and process antigenic HER2 oncoprotein to present the cognate antigen peptides to both $CD4^+$ T cells and/or $CD8^+$ T cells to elicit host immune responses against HER2 expressing tumors. We also examined the usefulness of DCs pretreated with CHP-HER2 complex for the purpose of immune therapy.

Materials and methods

Mice. In all experiments, 6 to 8-week-old female BALB/c mice purchased from Shizuoka Animal Laboratory Center (Shizuoka, Japan) were used and maintained at the Animal Center of Mie University School of Medicine, Tsu, Japan.

Tumor cell lines. CMS 7 and CMS17 are 3-methylcholanthrene-induced fibrosarcoma cell lines of BALB/c mouse origin (3). These lines were transfected with full length cDNA of human HER2 and designated CMS7HE and CMS17HE as described (12).

Antibodies. Anti-CD3 (145-2C11), anti-L3T4/CD4 (GK1.5), anti-Lyt2.2/CD8 (19/178), anti-H-$2K^d$ (20-8-4), anti-I-A^d (MKD-6), anti-B220/CD45R (RA3-3A1/6.2) monoclonal antibodies were produced as described (19). Anti-ICAM-1/CD54 (YN1/1.7.4), anti-LFA-1 (KBA), anti-B7-1/CD80 and anti-B7-2/CD86 monoclonal antibodies were purchased from Pharmingen, USA. Anti-DEC-205 (NLDC-145) was a generous gift from Dr Kraal, Leiden, The Netherlands.

Preparation of cholesteryl group-bearing polysaccharide nanoparticles. CHP-108-0.9 was exactly the same as those used in previous work (20,21). Pullulan (MW = 108,000) was substituted by 0.9 cholesteryl moieties per 100 glucose units of pullulan. An appropriate amount of CHP was dissolved in DMSO and dialyzed against PBS (150 mM, pH 7.9). After dialysis, the suspension was sonicated using a probe type sonifier (TOMY, UR-200P, Tokyo, Japan) at 40 W for 10 min. The obtained suspension was filtered through three types of membrane filters (Super Acrodisc 25, Gelman Science, pore size: 1.2 mm, 0.45 mm, and 0.2 mm) to make the particles and to remove dust. Finally, an optically clear suspension was obtained. The cholesteryl group-bearing polysaccharides formed nanoparticles by self-aggregation in diameter of 20-30 nm (20,21).

Preparation of complexes between HER2 protein and cholesteryl group-bearing polysaccharides. The HER2 derived protein described above was dissolved in 6 M urea. The protein solution (2.0 mg/ml) was mixed with 2.1 ml of a suspension of cholesteryl-bearing polysaccharides (5.7 mg/ml) at room temperature, resulting in the formation of a CHP-HER2 complex (CHP: 5.0 mg/ml, protein: 0.25 mg/ml, 0.75 M urea) (6,7). CHP-carbonic anhydrase II (CAB, Sigma) complexes were prepared as control using the same method (20-22).

Preparation of T cells and DCs. BALB/c mice were subcutaneously immunized twice with CHP-HER2 complexes (20 μg of truncated HER2 protein and 400 μg of CHP) or three times with mitomycin C treated CMS17HE ($2x10^6$) at one week interval. Spleen cells were obtained one week after the last immunization. For preparation of T cell subpopulations, spleen cells were enriched by using nylon fiber columns (23) followed by the treatment with anti-Lyt2.2 (CD8) mAb or anti-L3T4 (CD4) mAb and low toxicity rabbit complement (Cedarlane, Ontario, Canada) to obtain $CD4^+$ T cells and $CD8^+$ T cells, respectively. Bone marrow-derived DCs were prepared from normal BALB/c bone marrow as described by Inaba *et al* (24) with minor modifications. Briefly, single bone marrow cell suspensions were obtained from femurs and tibias, then depleted from lymphocytes, granulocytes and Ia^+ cells by using a mixture of mAbs (anti-CD4, anti-CD8, anti-B220/CD45R and anti-Ia) for 45 min on ice, followed by an incubation with low toxicity rabbit complement for 30 min at 37°C. Cells were resuspended at a concentration of 10^6 cells/ml of RPMI 1640 medium supplemented with 10 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) and were plated at 3 ml per well of 6-well plates. Floating cells were removed on day 3 and day 5 of culture by gentle pipetting and fed with fresh medium. On day 7 of culture, non-adherent and slightly adherent cells were collected for experiments. The phenotype of DCs were analyzed by FACScan flow cytometry.

T cell proliferation assay. Nylon fiber-purified suspensions of $CD4^+$ T cells or $CD8^+$ T cells from immunized mice were plated into 96-well U bottom microtiter plates at $3x10^5$ cells/well and used as responder cells. DCs pretreated with CHP-HER2 complex, CHP-CAB complex or HER2 protein only (75 μg protein/ml medium) for 3 h, or untreated DCs were added as stimulator cells at R:S ratio of 40:1 followed 18 h culture. After incubation in RPMI 1640 supplemented with 10% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol at 37°C in 5% CO_2 atmosphere for 90 h, cells were labelled with 1 μCi/well ^{3}H-thymidine during the last 18 h of culture and proliferation was determined by microplate scintillation counter. Results are presented as the mean of duplicate (25,26).

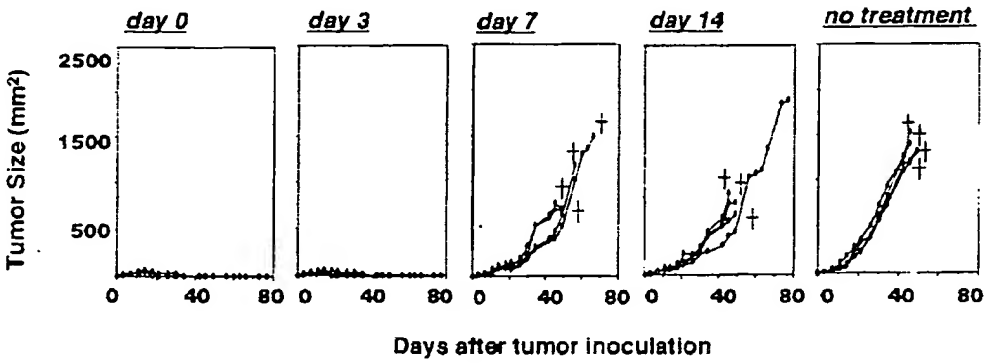

Figure 1. Therapeutic effect of the CHP-HER2 vaccine. BALB/c mice were challenged with $2x10^6$ CMS7HE subcutaneously and weekly given CHP-HER2 complex containing 20 µg of protein starting on the day of challenge, or 3, 7 or 14 days later. Each group consisted of four mice, a line represents a single mouse.

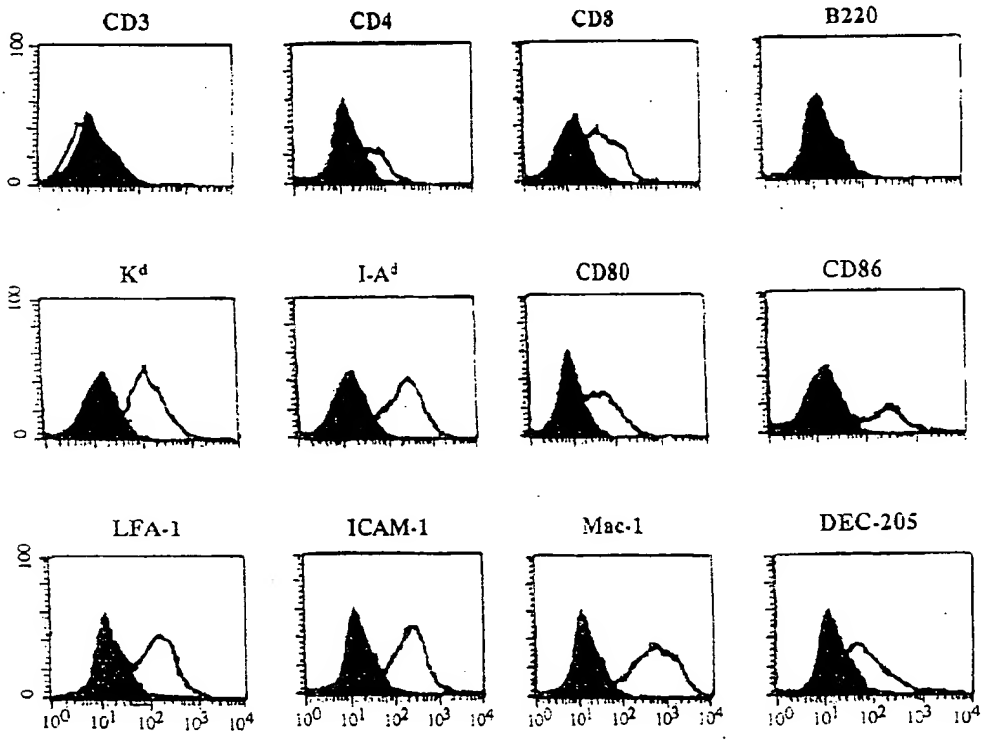

Figure 2. The phenotype of DCs. Bone marrow cells were depleted with anti-CD4, anti-CD8, anti-I-A^s and anti-B220/CD45R, thereafter cultured in the presence of rmGM-CSF (1000 unit/ml). On day 7, the culture cells were harvested and stained with the indicated monoclonal antibodies. The filled histograms represent the isotype controls. The figure shows the results of a representative experiment.

Results

Immunization with the CHP-HER2 complex is therapeutically effective against HER2 expressing tumors. BALB/c mice inoculated with $2x10^6$ HER2 expressing CMS7HE tumor cells were given weekly immunization of 20 µg protein of CHP-HER2 complex starting on the day of the challenge or 3, 7, or 14 days after the tumor challenge, respectively. Complete tumor rejection was observed when the immunization was initiated either on the day of tumor challenge or on day 3 after primary tumor challenge (Fig. 1). When the immunization was started 7 days or 14 days after the tumor inoculation, only marginal suppression of tumor growth was observed without complete rejection.

DCs can incorporate CHP-HER2 complex and specifically stimulate $CD8^+$ T cells and $CD4^+$ T cells. We questioned whether bone marrow-derived DCs could incorporate CHP-HER2 complex and stimulate T cells by providing the cognate target peptides. Bone marrow-derived DCs were prepared by culturing bone marrow cells in the presence of rmGM-CSF as described in Materials and methods. The phenotypic

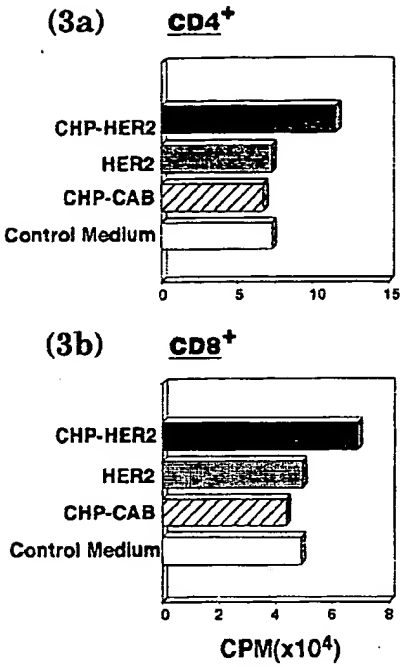

Figure 3. Proliferation of spleen T cells evaluated by ^{3}H-TdR uptake assay. BALB/c mice were immunized subcutaneously three times weekly with $2x10^6$ CMS17HE. Responder CD4+ T cells and CD8+ T cells were prepared from BALB/c mice spleen cells one week after the last immunization. DCs cultured with CHP-HER2 complex, CHP-CAB control complex, HER2 alone or control medium were used as APCs at R:S ratio of 40:1. Both CD4+ T cells (a) and CD8+ T cells (b) showed the strongest response to DCs treated with CHP-HER2 complex.

characteristics of generated DCs are presented in Fig. 2. BALB/c mice were immunized 3 times with subcutaneous injection of $2x10^6$ mitomycin C treated CMS17HE at a weekly interval. One week after the last immunization, CD4+ T cell and CD8+ T cell subpopulations were prepared. DCs cultured with CHP-HER2 complex, a control CHP-CAB complex, HER2 protein alone for 3 h, or untreated DCs were used as antigen presenting cells to stimulate T cells. Both CD4+ T cells and CD8+ T cells showed a significantly stronger response to DCs treated with CHP-HER2 complex than to DCs treated with CHP-CAB complex or HER2 protein alone, or to DCs without prior treatment (Fig. 3). A similar type of experiment was performed with T cells from BALB/c mice immunized twice with CHP-HER2 complex or HER2 protein alone subcutaneously at a weekly interval. The proliferative response of T cells was examined by stimulating them with DCs pretreated with CHP-HER2 complex, CHP-CAB control complex, HER2 alone, or without treatment. In both groups immunized with CHP-HER2 complex and HER2 protein alone, CD4+ T cells displayed the strongest response to CHP-HER2 pretreated DCs (Fig. 4a and b), similar to the results in CMS17HE immunized mice (Fig. 3). CD4+ T cells also responded moderately to DCs pretreated with HER2 protein alone when compared to DCs pretreated with CHP-CAB complex or without treatment. In contrast, CD8+ T cells, whether derived from CHP-HER2 complex immunized animals or HER2 immunized animals, responded only to DCs pretreated with CHP-HER2 complex (Fig. 4c and d). These results clearly show that DCs can incorporate CHP-HER2 complexes efficiently and present cognate peptides to both CD4+ T cells and CD8+ T cells after appropriate processing.

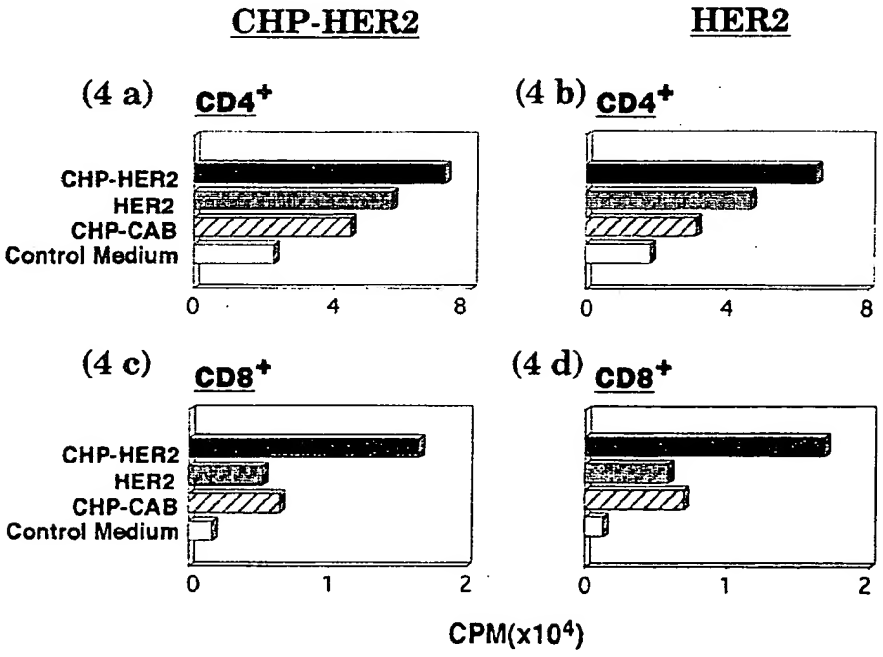

Figure 4. Bone marrow derived DCs demonstrate a potent APC function. DCs pretreated with CHP-HER2 complex, CHP-CAB complex, HER2 alone, or control medium were used as stimulator cells. Responder CD4+ T cells and CD8+ T cells were obtained from nylon wool-purified spleen cells of mice immunized with CHP-HER2 complex (a and c) or HER2 protein (b and d). ^{3}H-TdR proliferation assay was performed. CD4+ T cells (a and b) showed the strongest response to CHP-HER2 complex pretreated DCs, whereas CD8+ T cells (c and d) significantly responded only to CHP-HER2 complex pretreated DCs.

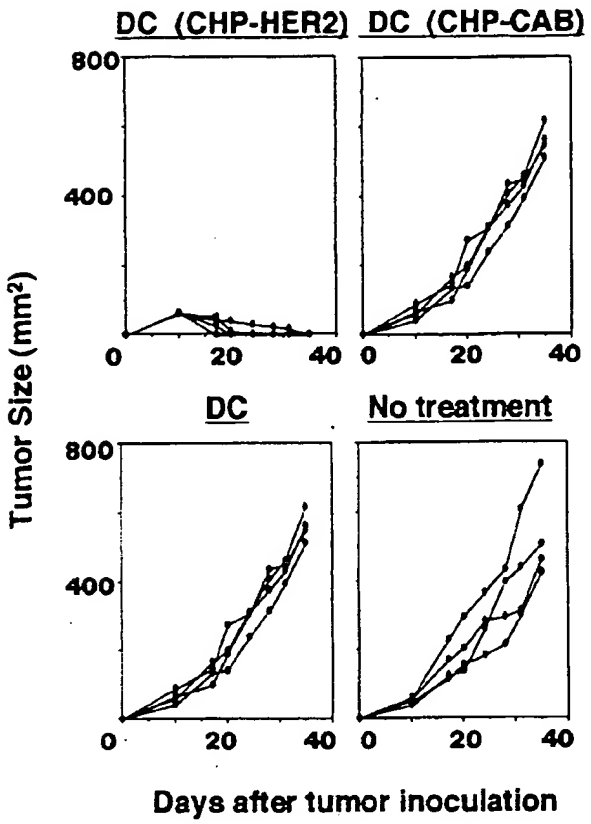

Figure 5. The therapeutic effect of DCs pretreated with CHP-HER2 complex. BALB/c mice were challenged with $2x10^6$ CMS7HE subcutaneously. Vaccination with $4x10^5$ DCs pretreated with CHP-HER2 complex, CHP-CAB control complex or without prior treatment was started on day 10 after tumor challenge and continued on a weekly basis. Strong tumor suppression was only observed in the group of mice vaccinated with CHP-HER2 complex pretreated DCs. Four mice were used for each experimental group, a line represents a single mouse.

Experimental cell therapy using DCs pretreated with CHP-HER2 complex. We further examined whether DCs treated *ex vivo* with CHP-HER2 complex could be used as vaccine against HER2 expressing tumor cells. $2x10^6$ CMS7HE cells were inoculated subcutaneously into BALB/c mice. 10 days after inoculation, vaccination with $4x10^5$ DCs pretreated with CHP-HER2 complex or CHP-CAB control complex, or DCs without treatment was started subcutaneously at a weekly basis. As shown in Fig. 5, in the group of mice vaccinated with CHP-HER2 complex pretreated DCs, complete eradication of tumor was observed in all the 4 mice. In contrast, tumor growth in mice of groups either treated with DCs pretreated with CHP-CAB control complex or DCs alone was similar to the tumor growth observed in mice without vaccination.

Discussion

An exogenous soluble protein antigen, when administered to hosts, is in general inefficient in inducing $CD8^+$ CTL, since it hardly enters the MHC class I pathway being rather internalized into endosomes and MHC class II presented (27-32). We reported that soluble truncated hybrid protein of gag and env of human T lymphotropic virus type I could induce specific $CD8^+$ T cell-dependent immunity when reconstituted into mannan derivative-coated liposomes (30,33). Considering the increasing evidence for receptors that can specifically bind polysaccharide on antigen presenting cells such as DCs and macrophages, we designed a novel and simple protein delivery system by utilizing CHM or CHP complexed with a truncated HER2 protein containing the peptide HER2 p63 that can induce $CD8^+$ CTLs against HER2 expressing tumor cells with K^d restriction. We demonstrated that the truncated protein containing 147 N-terminal amino acids of HER2 complexed with CHM or CHP can induce HER2 p63 specific anti-tumor $CD8^+$ T cells (4). In the present study we primarily questioned whether bone marrow-derived DCs can incorporate CHP-HER2 complex and process the molecules in order to present the cognate target epitopes to T cells. In addition we questioned whether thus processed DCs are a useful tool for immunotherapy of HER2 expressing tumors.

The CHP-HER2 complex was revealed to be as therapeutically potent as the CHM-HER2 complex on which we reported previously (4). Mice *in vivo* immunized by HER2 expressing CMS17HE tumor cells can generate both $CD4^+$ T cells and $CD8^+$ T cells specifically reactive with DCs pretreated with CHP-HER2 complex. When mice were immunized with either CHP-HER2 complex or HER2 protein alone, they were also able to generate both $CD4^+$ T cells and $CD8^+$ T cells specifically reactive with DCs pretreated with CHP-HER2 complex. It is interesting that for these animals either immunized with CHP-HER2 complex or HER2 protein alone, DCs pretreated with CHP-HER2 complex but not HER2 protein alone could strongly stimulate specific $CD8^+$ T cells *in vitro* as shown in Fig. 4. These results clearly show that DCs, whether pretreated with CHP-HER2 complex or HER2 protein alone can incorporate and process the antigen peptides and finally present them to $CD4^+$ T cells. However, they incorporated and processed antigenic protein in order to sufficiently present the cognate peptides to $CD8^+$ T cells only when they were pretreated with CHP-HER2 complex but not with HER2 protein alone. These results support our previous findings that a hydrophobized polysaccharide complexed with antigenic protein could efficiently generate $CD8^+$ T cell-dependent immunity either measured *in vitro* as CTL activity or *in vivo* as tumor rejection. The exact molecular mechanisms of how hydrophobized polysaccharide protein complexes are MHC class I presented, remains to be elucidated. Carbohydrate receptors on the cell surface of antigen presenting cells are one of possible explanations. DEC-205 and mannose receptor (MR) probably accept various polysaccharides as binding partners (34,35), that are structurally similar. Both tested substances mannan (4) and pullulan may fit to the binding sites of DEC-205 and/or MR. Since DEC-205 positive (Fig. 2) DCs were cultured and thereafter pulsed with CHP-HER complexe, the binding and internalization of the complexe is much more probable in our vaccination protocol than in the case of the administration of soluble protein only, which constitutes the beneficial effect for antigen presentation with DCs. The current analysis clearly indicates that there might be antigen

peptide(s) to be recognized by $CD4^+$ T cells, in addition to a K^d binding HER2 p63 peptide recognized by $CD8^+$ T cells (12) in this truncated HER2 protein. This notion is also supported by the evidence in our previous report that immunization with CHM-HER2 complex could elicit extremely high titers of IgG antibodies against the truncated HER2 protein suggesting an indispensable role of $CD4^+$ helper T cells (4). The characterization of the precise amino acid sequence of the peptide recognized by $CD4^+$ T cells is ongoing.

Having established that bone marrow-derived DCs can efficiently stimulate both $CD4^+$ T cells and $CD8^+$ T cells (13-18), we examined their usefulness for immunotherapy of HER2 expressing tumors. As shown in Fig. 5, treatment of mice inoculated with CMS7HE 10 days prior to immunization, obvious suppression of tumor growth was observed in the group utilizing DCs pretreated with CHP-HER2 complex. Non-specific adjuvant effect of CHP seems to be unlikely because DCs treated with CHP-CAB control complex showed no effect for tumor suppression when compared with mice without any immunization. It is of particular interest that in mice immunized with CHP-HER2 complex pretreated DCs, there was complete tumor eradication observed in all the 4 mice. In our experience, immunization either with CHP-HER2 complex or CHM-HER2 complex, complete tumor suppression was possible only when we initiated it sooner than 4 days following tumor inoculation. The present data strongly suggest that CHP-HER2 complex can be effectively used as a cancer vaccine in concert with bone marrow-derived DCs for immunological cell therapy.

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GENERATION OF IN VITRO AUTOLOGOUS HUMAN CYTOTOXIC T-CELL RESPONSE TO E7 AND HER-2/Neu ONCOGENE PRODUCTS USING EX-VIVO PEPTIDE LOADED DENDRITIC CELLS

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1. INTRODUCTION

Cytotoxic T-cells (CTL) have been shown to be capable of causing tumour-specific cell lysis when primed with tumour-specific antigenic peptides presented in conjunction with haplotype matched cell surface MHC class I molecules. For this approach to be successful for immunotherapy of tumours in vivo, it is essential that it is shown to be capable of generating adequate numbers of tumour-specific CTL either in vivo for active immunisation, or ex vivo for adoptive transfer therapy. A powerful means for ex vivo expansion of CTL has been recently shown to be antigen loaded autologous dendritic cells (DC)[1]. It has also become possible to generate large numbers of these cells from bone marrow or blood derived precursor cells cultured in the presence of GM-CSF and IL4[2]. Furthermore, murine studies have confirmed the efficacy of these cells to evoke significant ex vivo and in vivo responses against tumour specific antigenic peptides, in particular, HPV 16 E7 and HER-2/neu oncogene products[3], thereby creating the possibility of using these antigenic targets[4] for effective immunotherapy of gynaecological cancers such as cervical and ovarian carcinomas in which these tumour associated antigens are expressed with 93%[5] and 30%[6] frequency, respectively.

Dendritic Cells in Fundamental and Clinical Immunology
edited by Ricciardi-Castagnoli, Plenum Press, New York, 1997

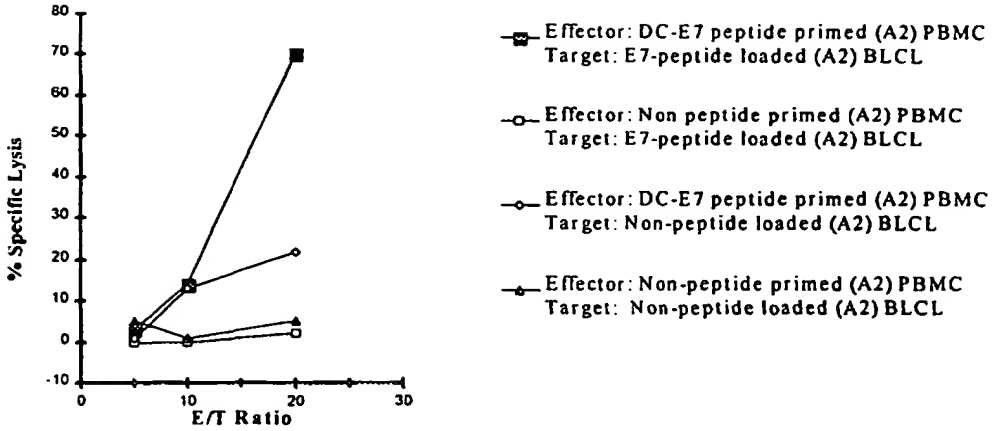

Figure 1. CTL responses using E7 loaded DC.

2. AIM

To assess the ability of ex vivo E7 or HER-2/neu peptide loaded DC to elicit adequate in vitro peptide specific CTL responses against autologous haplotype matched cell targets, as a feasibility study prior to clinical trials.

3. MATERIALS AND METHODS

The autologous dendritic cells were prepared from individual patients using the in vitro culture method of Romani et al (1994) as outlined in table 1. Their overall yield, morphology, immunophenotypology and FACS analysis based cell surface phenotype after 8 days in culture were found to be as described in in tables 2 and 3 and figures 4, 5 and 6 respectively.

The 8 day DC were loaded with 3 different varieties of peptides the sequences of which are shown in table 4. Individual peptide loaded DC were used to prime CTL using

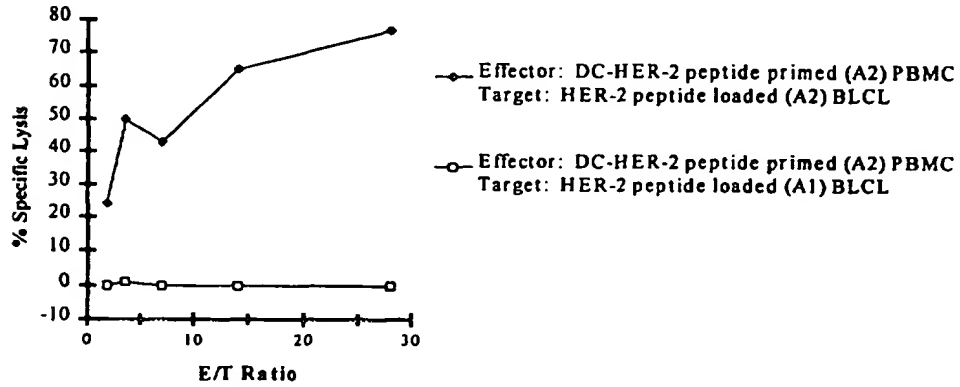

Figure 2. CTL responses using HER-2/neu loaded DC.

Table 1. Preparation of autologous dendritic cells

1. Isolation of peripheral blood monocytes using nycoprep 1.068 gradient centrifugation.
2. Lightly adherent fraction cultured with GMCSF (800 Iu/ml) + IL4 (500 Iu/ml) for 7 - 9 days.
3. Harvest poorly adherent fraction.
4. Identify DC using morphological (figure 1), immunocytochemical (figure 2) and FACS (figure 3) analysis, and determine yield.
5. Evaluate antigen presenting and T-cell priming ability of DC.

Table 2. Dendritic cell yields

Case	% Yield	Initial cell no./ 20 ml blood	Dendritic cell no. (day 9).
1	56%	1.5×10^6	8.3×10^5
2	42%	2.5×10^6	10.8×10^5
3	20%	2.8×10^6	5.6×10^5
4	25%	1.8×10^6	4.6×10^5
5	26%	1.9×10^6	5.1×10^5
6	16%	3.1×10^6	5.0×10^5
7	17%	2.3×10^6	4.0×10^5

1 - 2: Patient receiving radiotherapy for cervical cancer.
3 - 7: Healthy Volunteers.

the method outlined in table 5. The peptide primed CTL were used in CTL cytotoxicity assay (see table 6) which made use of EBV transformed B-cells (see table 7) as the target.

4. RESULTS

The strength and specificity CTL responses obtained using E7 HER-2/neu and flu peptides are graphically represented in Figures 1, 2 and 3. All these peptides were able to cause significant specific CTL responses at 20:1 effector : target ratios.

Table 3. Cell surface phenotype

	Case						
Marker	1	2	3	4	5	6	7
CD80 (B7.1)	+	+	++	+	+	+	+
CD86	+++	++	++	+++	+++	++	+++
HLA-DR (Class II)	++++	+++	+++	+++	+++	+++	+++
CD1a (Class I-like molecules)	+	±	+	±	+	±	+
CD54 (ICAM-1)	+++	++	++	+++	++	++	++
CD68 (monocytes, marophages)	±	+	±	+	+	±	+
CD20 (B-cells)	+2%	+4%	+5%	+7%	+7%	+2%	+2%

1 - 2: Patients receiving radiotherapy for cervical cancer
3 - 7: Healthy Volunteers

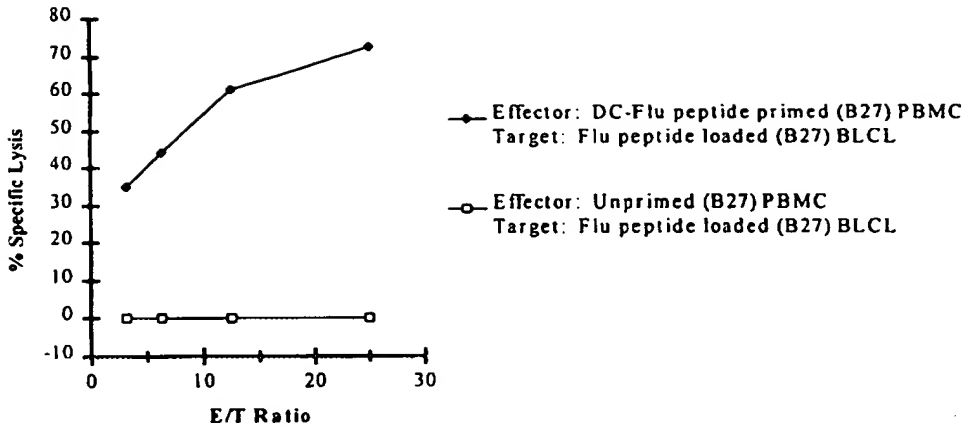

Figure 3. CTL responses using Flu loaded DC.

5. DISCUSSION

It is likely that for peptide-based immunotherapies to be successful that sufficient numbers of tumour specific CTL need to be recruited. Tumour specific CTL may be expanded in vivo in the human by immunisation of the tumour antigen. Borysiewicz and colleagues detected HPV specific CTL[7] in one of three evaluable patients with advanced cervical cancer after immunisation with a live recombinant vaccinia virus expressing the E6 and E7 proteins of the HPV16 and 18 virus. An alternative means of indirectly expanding tumour specific CTL in vivo which may prove to be suited to the clinical situation is to infuse ex vivo tumour antigen peptide pulsed autologous DC as anti-cancer therapy[8]. This approach is supported by recent murine studies[3,9]. In one tumour modal the murine C3 sarcoma which presents HPV16 E7 antigen treatment of animals bearing established

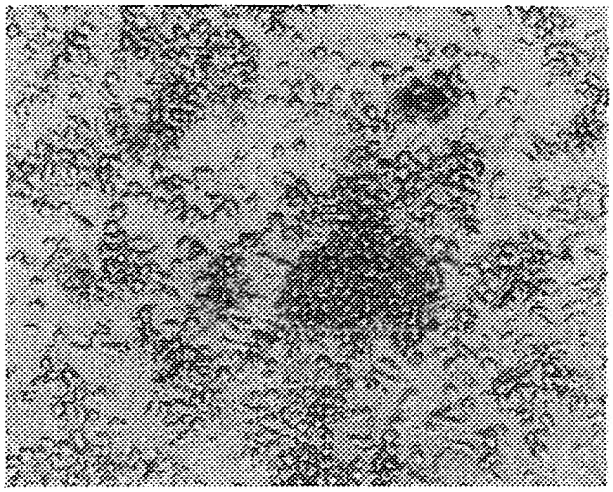

Figure 4. Morphological features of DC: day 8 – case 2.

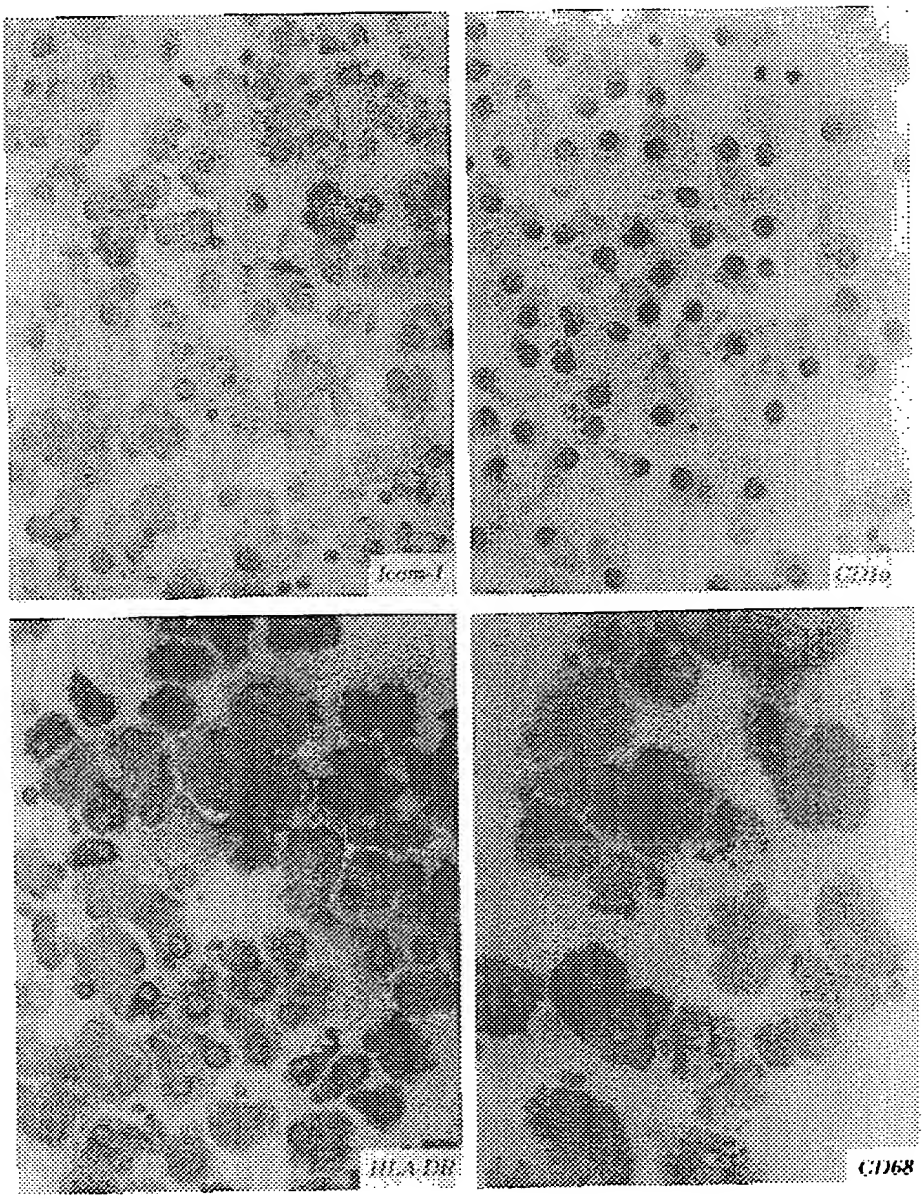

Figure 5. Immunophenotypic features of DC: day 8 – case 4.

macroscopic tumour (up to 1cm^2) with DC loaded with HPV16 E7 peptide resulted in sustained complete eradication of tumour masses in 80% of mice[3].

The present study further confirms that it is possible to induce Class I restricted CTL responses using ex vivo peptide loaded dendritic cells obtained from human peripheral blood progenitors cultured with lymphokines IL4 and GMCSF[2]. Significant numbers of DC have been produced from peripheral blood of normal individual cancer patients. Specific CTL responses MHC Class I have been induced using a variety of peptides including flu, HER-2/neu and HPV16 E7. The latter two antigens may represent feasible tar-

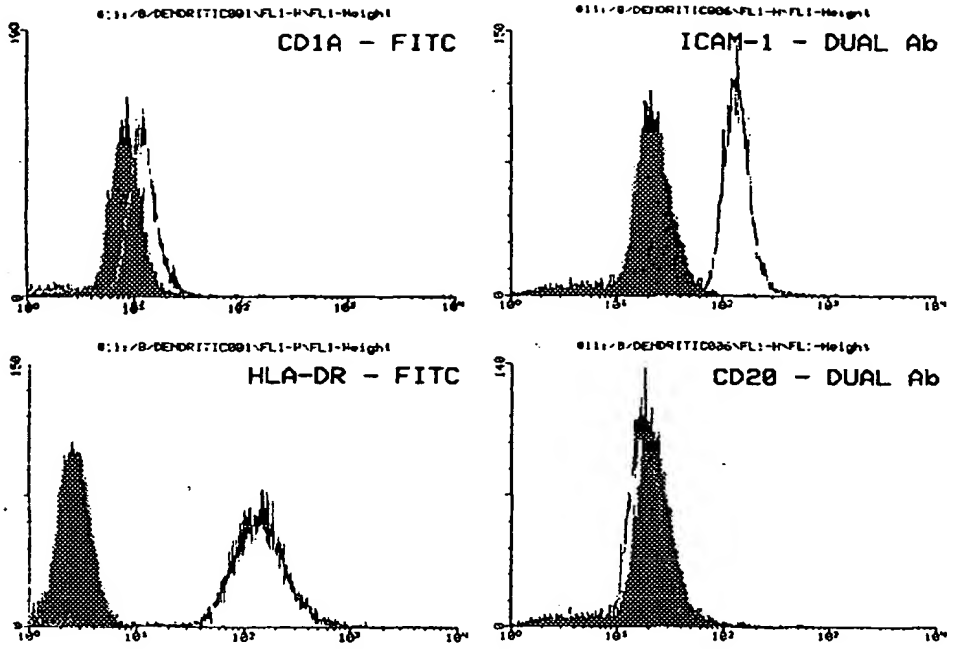

Figure 6. FACS characteristics of DC: day 8 – case 2.

Table 4. Target peptides sequences

Code	Class I	aa	Position
HER-2/neu	A2 specific	KIFGSLAFL	369 - 377
HPV16 E7	A2 specific	YMLDLQPETT	11 - 20
Flu	B27 specific	SRYWAIRTR	383 - 391

Table 5. Antigen specific priming of CTL

1. Load DC with peptide + B2 - microglobulin.
2. Irradiate peptide loaded DC.
3. Co-culture peptide loaded DC with autologous PBMC.
4. Culture in human serum + IL7 for 12 days and restimulate PBMC with peptide loaded DC after 7 days.
5. Add IL2 to culture days 12 - 14.
6. Harvest for CTL day 15.

Table 6. CTL cytotoxicity assay

1. Label + pulse target cells (autologous EBV transformed B cells) with 51Cr and synthetic peptide.
2. Mix target cells with autologous effector cells.
3. Incubate 4 - 6 hours.
4. Count radioactivity.

Table 7. Preparation of EBV transformed B cells as autologous cell targets

1.	Take 10ml of blood into tubes containing preservative free heparin
2.	Separate PBMCs using Histopaque as a density-gradient
3.	Pellet cells and add 1.0ml of EBV (B95.8 cell line) supernatant
4.	Incubate for 1 hour at 37°C
5.	Wash cells with RPMI
6.	Pellet cells resuspend in RPMI + 20% FCS. PHA (1% v/v) and 1% penicillin/streptomycin
7.	Aliquot into wells of a 24 well plate at 1 - 2 × 10^6 cells/well
8.	Culture at 37°C
9.	After two weeks foci of B cells should be visible
10.	Split cells 1:2 as necessary and feed twice weekly by removing half the supernatant and replacing it with fresh medium without PHA

gets for immunotherapy of cervical and ovarian cancer respectively. These data further support the rationale for testing autologous tumour antigen peptide primed DC as a potential means of immunotherapy in gynaecological cancer in clinical study.

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Induction of Antitumor Immunity Using Bone Marrow-Generated Dendritic Cells

Angel Porgador,[1] David Snyder, and Eli Gilboa[2]*

We have previously shown that bone marrow-generated dendritic cells (DC) pulsed with a class I-restricted peptide are potent inducers of CD8$^+$ CTL. In the present study we have investigated whether bone marrow-generated DC are capable of inducing antitumor immunity. We show that a single immunization with DC pulsed with OVA peptide was highly effective in eliciting a protective immune response against a challenge with tumor cells expressing the OVA gene (E.G7-OVA), more so than immunization with irradiated E.G7-OVA cells, OVA peptide-pulsed RMA-S cells, or free OVA peptide mixed with adjuvant. The addition of free OVA protein to day 4 or day 7 bone marrow cultures, but not to day 9 mature DC, was also effective in eliciting CTL and engendering antitumor immunity, but was less effective than peptide-pulsed DC. Induction of CTL and antitumor immunity by bone marrow-generated DC pulsed with the class I-restricted OVA peptide correlated with the expression of syngeneic MHC class II molecules on the DC. This and the fact that induction of tumor immunity was dependent on CD4$^+$ T cells suggest that in vivo priming of CTL and induction of antitumor immunity by bone marrow-generated DC also require the presentation of MHC class II-restricted epitopes and activation of CD4$^+$ T cells. This observation has potentially important implications to the use of peptide-pulsed DC in clinical immunotherapy. *The Journal of Immunology,* **1996, 156: 2918–2926.**

CD8$^+$ CTL are an important effector arm in antitumor immunity (1, 2). The existence of specialized APC that are responsible for the presentation of Ag to naive CD8$^+$ T cells was postulated from observations that host MHC-restricted CTL could be primed in vivo to Ag that was introduced on MHC-disparate cells (3) and from transplantation studies suggesting that only a subset of allo-MHC donor cells, called passenger leukocytes, were responsible for the induction of an immune response and rejection of the mismatched tissue (4). Studies exploring the mechanism of action of IL-2 and GM-CSF[3]-secreting tumor vaccines have also suggested that priming of an MHC class I-restricted antitumor response required the transfer of Ags from tumor cells to a host-derived cell for presentation to CD8$^+$ CTL (5, 6).

A wealth of evidence suggests that the host-derived cells that specialize in presenting Ag to naive T cells, called professional APC, are dendritic cells (DC) of hemopoietic origin (7). Consistent with this idea, several studies have documented the exceptional ability of DC to stimulate naive CD4$^+$ and CD8$^+$ T cells in vitro and in vivo. For example, DC pulsed with protein in the presence of lipid (8) or transfected with DNA (9) are capable of eliciting a primary CTL response in vitro, and inoculation of mice with small numbers of allogeneic DC (10) or with peptide-pulsed DC (11) induces a strong CTL response in vivo.

Immunization with DC loaded with tumor Ags may, therefore, represent a potentially powerful method of inducing antitumor immunity. Consistent with this hypothesis, Shimizu et al. (12) have shown that APC-enriched splenocytic fractions from BALB/c mice pulsed with tumor fragments were able to protect mice against a tumor challenge. Similarly, two studies have shown that immunization with DC-enriched preparations pulsed with tumor fragments or with defined tumor Ags induced protective immunity in mice (13, 14).

DC are present in trace amounts in many tissues of lymphoid and nonlymphoid origin, requiring the use of tedious and time-consuming procedures to isolate relatively small numbers of cells. Inaba et al. (15) have developed a protocol to generate large numbers of DC from the bone marrow of mice by culturing cells in the presence of GM-CSF. The bone marrow-generated DC, like their mature counterparts, were highly effective in stimulating quiescent T cells in allogeneic MLR and presented mycobacterial Ags to unprimed CD4$^+$ T cells in vivo (16). We have recently shown that DC generated from the bone marrow of mice pulsed with an MHC class I-restricted peptide were also potent inducers of CTL in vivo (17).

The objective of the current study was to assess the ability of bone marrow-generated DC to induce antitumor immunity. The main findings from this study were that immunization with DC pulsed with a tumor rejection Ag encoding an MHC class I-restricted epitope were highly effective in protecting mice against a challenge with tumor cells, and that induction of CTL and antitumor immunity was dependent on the expression of syngeneic MHC class II molecules on the DC. The implications of these findings to the use of DC-based vaccines in human patients are considered.

Materials and Methods

Mice

Five- to six-week-old female C57BL/6 (H-2^b), C3H/He (H-2^k), B6.C-H2^{bm1}/By (H-2^{bm1}), B10.MBR/SxEg (H-2^{bq1}), and F$_1$(C57BL/6 × C3HeB/Fe) (H-2^b × H-2^k) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). F$_1$(B10.MBR/SxEg × C57BL/6) (H-2^{bq1} × H-2^b) were bred at the Duke University Medical Center vivarium (Raleigh, NC), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were used at 7 to 10 wk of age.

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[1] Recipient of a Sara Belk Gambrell fellowship from the Cancer Research Institute (New York, NY).

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[3] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage colony-stimulating factor; DC, dendritic cells; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate.

0022-1767/96/$02.00

Animals were maintained and treated accordingly to National Institutes of Health guidelines.

Cell lines and cell cultures

The tumor cell lines used were EL-4 (C57BL/6, H-2^b, thymoma) and E.G7-OVA (EL-4 cells transfected with the chicken OVA cDNA (18). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. E.G7-OVA cells were grown in medium containing 400 mg/ml G418 (Life Technologies, Grand Island, NY). EL-4 and E.G7-OVA were free of mycoplasma as tested by the *Mycoplasma* T.C. RNA detection kit (Jen-Probe, San Diego, CA).

DC generation from bone marrow cultures

The procedure used in this study was as described by Inaba et al. (15), with some minor modifications. Briefly, bone marrow was flushed from the long bones of the limbs and depleted of red cells with ammonium chloride. Bone marrow cells were depleted from lymphocytes, granulocytes, and Ia^+ cells using a mixture of mAbs and rabbit complement. The mAbs were 2.43 or 53-6.72 anti-CD8, GK1.5 anti-CD4, RA3-3A1/6.1 anti-B220/CD45R, B21-2 anti-Ia (TIB 210, 105, 207, 146, and 229, respectively; American Type Culture Collection, Rockville, MD) and RB6-8C5 anti-Gr-1 (kindly provided by DNAX, Palo Alto, CA). Cells were plated in six-well culture plates (10^6 cells/ml, 3 ml/well) in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 50 mM 2-ME, 10 mM HEPES (pH 7.4), 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 3.3 ng/ml recombinant murine GM-CSF (kindly provided by Amgen, Inc., Thousand Oaks, CA). On day 3 of culture, floating cells were gently removed, and fresh medium was added. On day 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were collected and replated in 60-mm petri dishes (10^6 cells/ml, 5 ml/dish). At 10 days of culture, nonadherent cells (DC) were removed for analysis and immunizations.

Peptides and proteins

OVA peptide (amino acids 257–264, SIINFEKL, H-2K^b restricted) (19) and mut1 peptide (FEQNTAQP, H-2K^b restricted) (20) were purchased from Research Genetics (Huntsville, AL). Peptides were synthesized by solid phase techniques with free amino and carboxyl ends and purified by reverse phase HPLC. Peptides were dissolved in serum-free IMDM-50 mM 2-ME and stored at −20°C. OVA protein (grade VI, Sigma Chemical Co., St. Louis, MO) was dissolved either in RPMI 1640/20 mM HEPES (pH 7.4)/50 mM 2-ME (for pulsing of DC with lipid-protein complexes) or in culture medium of DC (for pulsing of DC with free soluble protein) and stored at −20°C.

Antigen pulsing of DC

With peptide. Day 10 DC ($1–4 \times 10^6$) were washed once in IMDM-50 mM 2-ME and resuspended in 0.8 ml of the same medium containing 100 mg of peptide. After 3-h incubation at 37°C (with gentle shaking every 30 min), cells were washed twice in HBSS and resuspended in HBSS for injections.

With lipid-protein. Four hundred milligrams of OVA protein and 30 mg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Boehringer Mannheim, Indianapolis, IN) were incubated in 0.2 ml of RPMI 1640/20 mM HEPES (pH 7.4)/50 mM 2-ME for 15 min at room temperature, as described by Nair et al. (8). Day 10 DC ($1–4 \times 10^6$) were washed twice in RPMI 1640/20 mM HEPES (pH 7.4)/50 mM 2-ME and resuspended in 0.8 ml of the same medium with the DOTAP-OVA complexes for 25 min at 37°C. Three milliliters of the same medium with 6% FCS were added for an additional 1-h incubation. Cells were washed twice in HBSS and resuspended in HBSS for injections. Using a mixture of OVA protein and FITC-labeled unrelated protein (15/1) to follow the fate of DOTAP-complexed protein incubated with the bone marrow-generated DC, >95% of the DOTAP-complexed protein was delivered into the cell (data not shown).

With free soluble protein. OVA protein (5 mg/ml) was added once to DC cultures on day 4 and again on day 7 to the washed and replated cells. On day 10, cells were washed twice in HBSS and resuspended in HBSS for injections.

CTL induction in vivo and cytotoxicity assays

Ag-pulsed DC on day 10 (1.5×10^5 to 9×10^3 cells/mouse; in 0.5 ml HBSS) were injected once i.v. or i.p. Spleens were removed from mice 7 to 9 days after inoculation. Splenocytes or nonadherent splenocytes (17) from the primed mice (2×10^6/ml) were cultured for 5 days in vitro with irradiated (200 Gy) E.G7-OVA cells (2×10^5/ml) in six-well culture plates (5 ml/well). The culture medium was NCTC 109 and RPMI 1640 (1/1, v/v) supplemented with 10% heat-inactivated FCS, 50 mM 2-ME, 20 mM HEPES (pH 7.4), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin. After 5 days of restimulation, graded doses of viable lymphocytes were plated in 96-well V-bottom culture plates (in triplicate) and cocultured for 4 h with europium-diethylenetriaminepentaacetate-labeled target cells (21). Europium-diethylenetriaminepentaacetate release was measured by time-resolved fluorescence (1232 Delfia Fluorometer; Wallac, Gaithersburg, MD). SEMs of triplicate cultures were <5% of the means.

In vivo depletion of T cell subsets

mAbs GK1.5 and 2.43 (see above) were used to deplete mice of $CD4^+$ and $CD8^+$ T cells, respectively, as described previously (5). Briefly, mice were injected i.p. at 3-day intervals with 100 ml of hybridoma ascites fluid, except for the first injection which was given i.v. with 150 ml. The depletion protocol was initiated 4 days before either immunization with DC or challenge with tumor cells. Specific depletion was 95 to 100%, as determined by flow cytometry (data not shown).

Tumor protection in vivo

Groups of mice ($n = 6$) were immunized once as described for CTL induction in vivo. Seven days postimmunization, mice were challenge with 1 or 2×10^7 E.G7-OVA cells s.c. in the scapular region. Mice were monitored on a regular basis for tumor growth and size. Measurements of tumor diameter at specified times after tumor challenge are displayed for each mouse (dot) and also as an average for the group (column). Mice with tumor diameters of 3 to 3.5 cm were killed.

Results

Induction of antitumor immunity by bone marrow-generated DC

We have previously shown that bone marrow-generated DC pulsed with an MHC class I-restricted peptide are potent inducers of CTL in vivo (17). In this study we tested whether DC presenting a tumor Ag are also capable of inducing protective immunity against a tumor challenge.

The tumor used in this study, E.G7-OVA, was derived from the EL-4 thymoma line (H-2^b haplotype) by transfection with the chicken OVA gene (18), which encodes a dominant H-2K^b-restricted CTL epitope and serves as the tumor rejection Ag against which an immune response is generated. In the experiment shown in Figure 1, *A* and *B*, bone marrow-generated DC from C57BL/6 mice (H-2^b) were pulsed with an eight-amino acid long peptide derived from chicken OVA protein (OVA peptide, amino acids 257–264), which encodes the H-2K^b-restricted epitope (19), or with the control peptide, mut-1, which also encodes an H-2K^b restricted epitope (20). The peptide-loaded cells were injected into the tail vein (i.v.) of C57BL/6 mice (1.5×10^5 cells/mouse), and 7 days later, the mice were challenged with 2×10^7 EL-4 or E.G7-OVA tumor cells. As shown in Figure 1, *A* and *B*, mice immunized with HBSS or mut-1 peptide-pulsed DC and challenged intrascapularly with either E.G7-OVA or EL-4 cells developed large tumors 10 days later. Likewise, mice immunized with OVA peptide-pulsed DC and challenged with EL-4 tumor cells also developed large tumors. However, when mice were immunized with OVA peptide-pulsed DC and challenged with E.G7-OVA cells, no tumors were detected 10 days postchallenge. Nevertheless, the protection achieved with OVA peptide-pulsed DC was not complete, since within 30 days postchallenge, tumors grew in five of six mice, albeit at a slower rate than that in animals in the control groups. Yet, at lower doses of tumor challenge, DC were able to induce a long-lasting protective immunity. When 1.5×10^5 DC/mouse were used and mice were challenged with 10^7 E.G7-OVA tumor cells, the following results were obtained: all six unimmunized mice and all six mice immunized with the control mut1 peptide-pulsed DC developed tumors within 10 days postchallenge, and mice were killed within 30 days when tumor

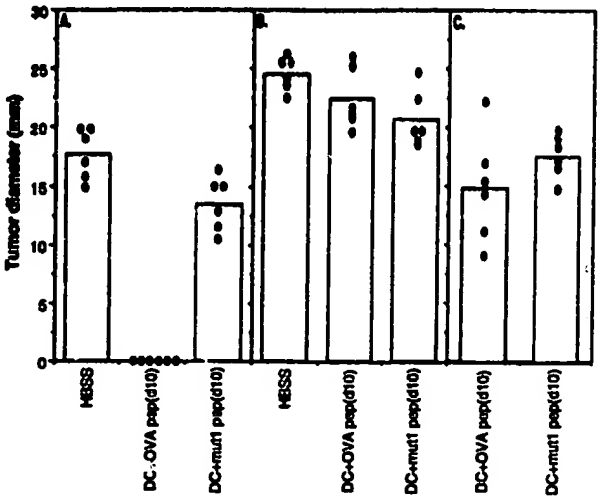

FIGURE 1. Induction of antitumor immunity in mice immunized with bone marrow-generated DC. C57BL/6 mice were inoculated once i.v. with either HBSS or 1.5×10^5 peptide-pulsed DC (mut1 or OVA peptide) as described in *Materials and Methods*. Mice were challenged intrascapularly with 2×10^7 live tumor cells 8 days postimmunization. Tumor measurements were taken 10 days postchallenge. *A*, DC were generated from C57BL/6 mice, and mice were challenged with E.G7-OVA cells. *B*, DC were generated from C57BL/6 mice, and mice were challenged with EL-4 cells. *C*, DC were generated from B10.MBR/SxEg mice, and mice were challenged with E.G7-OVA cells.

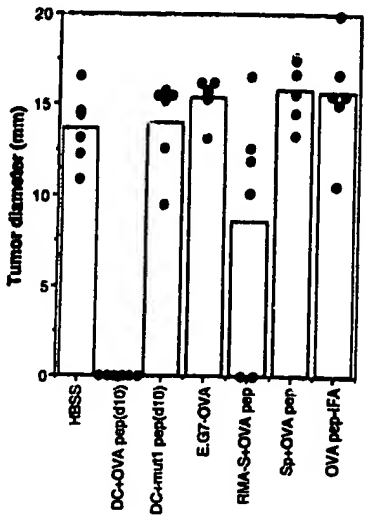

FIGURE 2. Comparative analysis of antitumor immunity induced by bone marrow-generated DC, splenocytes, RMA-S cells, irradiated E.G7-OVA tumor cells, and free peptide mixed with adjuvant. OVA peptide-pulsed cells (1.5×10^5 DC/mouse, 3×10^5 RMA-S cells/mouse, and 3×10^5 splenocytes/mouse) were inoculated i.v. RMA-S cells were incubated at 28°C for 48 h before peptide pulsing, which was performed at 37°C, and irradiated (50 Gy) before injection. E.G7-OVA cells (3×10^5 cells/mouse) were irradiated (50 Gy) and injected i.v. Ova peptide (100 mg/mouse) emulsified in IFA was injected s.c. Primed mice were challenged intrascapularly with 2×10^7 live E.G7-OVA cells 7 days postimmunization. Tumor measurements were made 11 days postchallenge.

size reached 3 to 3.5 cm in diameter. In contrast, only one of six mice immunized with OVA peptide-pulsed DC and challenged with 10^7 tumor cells developed tumors. Five of six mice remained free of tumor for 3 mo, at which time the experiment was terminated.

DC were also generated from the bone marrow of a congenic mouse strain B10.MBR/SxEg (H-2K^b, Ia^k). Therefore, DC from B10.MBR/SxEg mice should be able to present the OVA peptide to CD8$^+$ T cells in C57BL/6 mice. Surprisingly, however, OVA peptide-pulsed DC from B10.MBR/SxEg mice failed to induce protective immunity in C57BL/6 mice challenged with E.G7-OVA tumor cells (Fig. 1*C*; the experiments in Fig. 1, *A* and *B*, were performed nine times in various combinations; the experiment in Fig. 1*C* was performed once).

We have previously shown that peptide-loaded DC were highly effective in eliciting CTL in vivo compared with other methods of immunization (17). It was, therefore, of interest to asses in a similar manner the effectiveness of OVA peptide-loaded DC in eliciting antitumor immunity. Bone marrow-generated DC (1.5×10^5 cells/mouse), splenocytes (3×10^5 cells/mouse), or RMA-S cells (3×10^5 cells/mouse) were pulsed with OVA peptide and injected into syngeneic C57BL/6 mice. RMA-S cells were first incubated at 28°C for 48 h before pulsing with peptide and irradiated (5000 rad) before injection into mice. RMA-S cells, which are normally H-2K^b negative, reexpressed H-2K^b molecules on the cell surface following incubation with peptide (data not shown). For immunization with E.G7-OVA cells (3×10^5 cells/mouse), cells were irradiated (5000 rad), and for immunization with free OVA peptide (100 mg/mouse), peptide was emulsified in IFA and injected s.c. All immunizations were performed once without subsequent boosting. As shown in Figure 2, neither splenocytes, E.G7-OVA cells, nor free OVA peptide emulsified in IFA were able to elicit a measurable protective response. Some response was observed following immunization with OVA peptide-pulsed RMA-S cells, since two of six mice remained free of tumor at the time of measurement. In stark contrast, none of the mice immunized with OVA peptide-pulse DC develop tumors 11 days after inoculation of the E.G7-OVA tumor cells (this experiment was performed twice).

Requirement for MHC class II-restricted antigen presentation for CTL induction and antitumor immunity

Figure 1*C* shows that DC derived from the bone marrow of B10.MBR/SxEg (H-2K^b, Ia^k) mice failed to elicit protective immunity in C57BL/6 (H-2^b) mice challenged with E.G7-OVA tumor cells, despite the fact that B10.MBR/SxEg mice encode the H-2K^b restriction element. Thus, either the OVA peptide was not presented by K^b molecules in this experimental setting, B10.MBR/SxEg mice do not express a functional K^b product, or the MHC class I-restricted presentation by itself was insufficient for generating antitumor immunity. The experiment shown in Figure 3 was designed to address these issues. DC were generated from C57BL/6 (H-2^b) and C3H/He (H-2^k) mice as well as from the congenic strains B6.C-H2^{bm1}/By (H-2K^{bm1}, Ia^b) and B10.MBR/SxEg (H-2K^b, Ia^k), and used to immunize C57BL/6 mice or F_1 combinations with OVA peptide-pulsed DC. The presence of OVA-specific CTL was determined after restimulation of splenocytes with E.G7-OVA cells. Targets for CTL lysis were E.G7-OVA cells or RMA-S cells pulsed with OVA peptide and the corresponding control targets, EL-4 cells or RMA-S cells pulsed with the mut-1 peptide, respectively.

DC from C57BL/6 mice pulsed with OVA peptide elicited a strong CTL response in the syngeneic host as well as in F_1(C57BLxC3H) mice (Fig. 3*A*). It was previously shown (17), and is evident in Figure 3*A*, that bone marrow-generated DC induce also a high level of nonspecific lytic activity. Figure 3*B* shows that DC obtained from B6.C-H2^{bm1}/By mice are not capable of inducing CTL in either C57BL/6 or F_1(C57BLxC3H) mice.

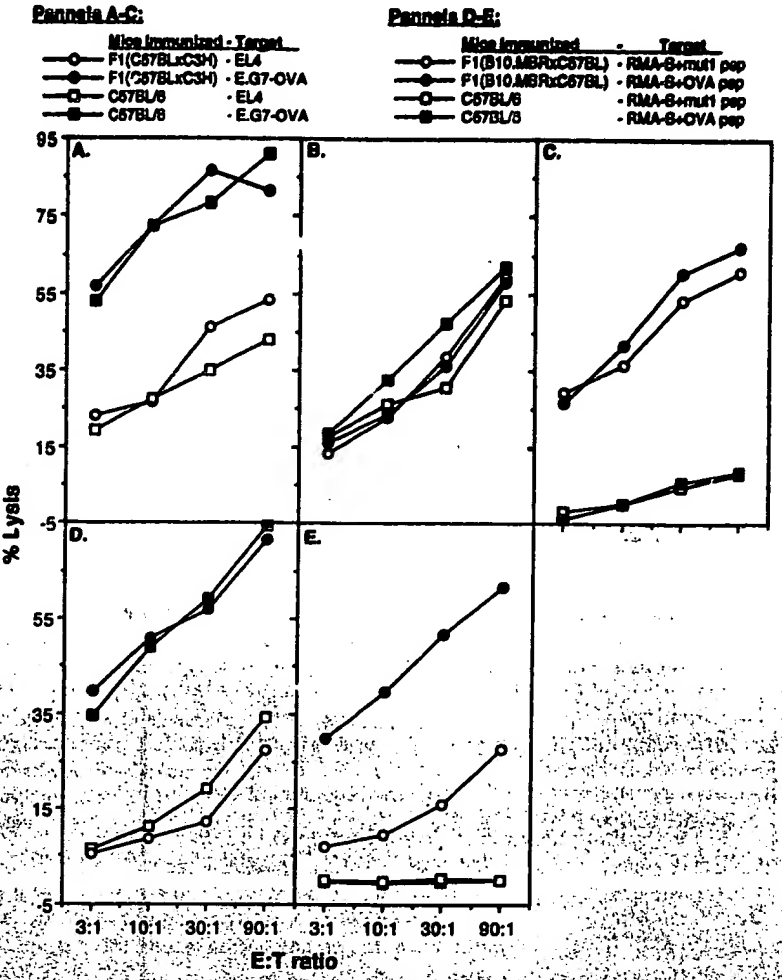

FIGURE 3. Induction of OVA-specific and nonspecific lytic activities by bone marrow-generated DC pulsed with OVA peptide. Day 10 DC were generated from C57BL/6 mice (*A* and *D*), B6.C-H2^{bm1}/By mice (*B*), C3H/He mice (*C*), and B10.MBR/SxEg mice (*E*); pulsed with OVA peptide on day 10; and inoculated i.p. into C57BL/6 mice or into F_1 combinations as indicated. Seven days later, splenocytes were isolated and restimulated with E.G7-OVA cells, and cytotoxic activity was measured against E.G7-OVA, EL-4, or RMA-S cells pulsed with 1 mM OVA or mut1 peptides, as described in *Materials and Methods*.

Table I. *The importance of H-2K^b and syngeneic class II expression on bone-marrow-generated DC for induction of OVA-specific and nonspecific lytic activity*

	Mice					
	DC		Immunized		Lysis	
Panel[a]	H-2K	Ia	H-2K	Ia	OVA-specific	Nonspecific
A	b	b	b	b	+	+
			b + k	b + k	+	+
a	bm1	b	b	b	−	+
			b + k	b + k	−	+
C	k	k	b	b	−	−
			b + k	b + k	−	+
D	b	b	b	b	+	+
			b	b + k	+	+
E	b	k	b	b	−	−
			b	b + k	+	+

[a] Data from Figure 3.

Since the major difference between C57BL/6 and B6.C-H2^{bm1}/By mice is a mutation in the H-2K^b molecule, this observation confirms the fact that the H-2K^b molecule is the restriction element used by the bone marrow-generated DC for presenting the OVA peptide to generate OVA-specific CTL. Note, however, that a high level of nonspecific lytic activity was, nevertheless, generated in this setting. As would be expected, DC from C3H/He mice were not able to generate K^b-restricted CTL (which recognize E.G7-OVA targets) in C57BL/6 or in F_1(C57BLxC3H) mice (Fig. 3*C*). Note, however, that immunization of F_1(C57BLxC3H) mice, but not of C57BL/6 mice, with C3H-derived DC generated a high level of nonspecific lytic activity, which is typically seen with bone marrow-generated DC. The experiment shown in Figure 3*D* is similar in design and results to the experiment shown in Figure 3*A*, except that OVA peptide-loaded RMA-S cells were used as targets instead of OVA protein-expressing E.G7-OVA cells, confirming that the bulk of the CTL activity generated in mice immunized with OVA peptide-pulsed DC is indeed directed against the epitope defined by the OVA peptide (amino acids 257–264). When DC from B10.MBR/SxEg mice were used to immunize C57BL/6 mice, neither OVA-specific nor nonspecific lytic activity was observed against OVA peptide- or control peptide-pulsed RMA-S cells (Fig. 3*E*), and similar results were obtained when E.G7-OVA and EL-4 cells were used as targets (data not shown). However, when F_1 (C57BLxB10.MBR) mice were immunized with the same DC, both OVA-specific and nonspecific lytic activities were generated, which proves that DC from B10.MBR/SxEg mice were competent APC.

The data from Figure 3 are summarized in Table I, which shows the relationship between the MHC haplotypes of the DC and the recipient mice, and the generation of OVA-specific and nonspecific lytic activities. The major conclusion from this experiment is that generation of specific CTL by bone marrow-generated DC pulsed with a class I-restricted peptide requires not only identity

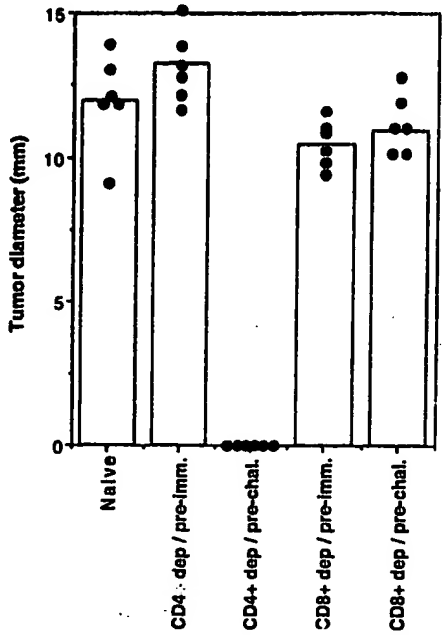

FIGURE 4. Involvement of T cell subsets in the rejection of E.G7-OVA tumor cells. C57BL/6 mice were depleted from $CD4^+$ or $CD8^+$ T cells either during immunization with DC pulsed with OVA peptide or during challenge with E.G7-OVA tumor cells. Mice were immunized once with 1.0×10^5 DC injected i.v. Eleven days postimmunization, mice were challenged with 10^7 E.G7-OVA cells injected intrascapularly. Tumor measurements were made 10 days posttumor challenge.

between the class I restriction elements of the APC and the responder $CD8^+$ T cells, the K^b allele in this instance, but also matching at the MHC class II locus. This is shown by the fact that DC from B10.MBR/SxEg (H-$2K^b$, Ia^k) mice were unable to induce CTL in C57BL/6 (H-$2K^b$, Ia^b) mice, but were able to do so in F_1 (C57BLxB10.MBR) (H-$2K^b$, Ia^b, Ia^k) mice (Fig. 3*E*).

A second conclusion suggested from the data shown in Figure 3 and Table I is that the nonspecific lytic activity typically seen when mice are immunized with bone marrow-generated DC (17) depends only on concordance between the APC and the responder T cells at the MHC class II locus, not at the MHC class I locus. For example, DC from C3H mice (H-2^k) generate nonspecific lytic activity when injected into C57BLxC3H (Ia^k, Ia^b), but not in C57BL/6 mice (Ia^b), whereas DC from B6.C-$H2^{bm1}$/By mice, which do not express a functional K^b allele, induce nonspecific lysis in C57BL/6 or F_1 mice. In neither case was OVA-specific CTL generated because of incompatibility at the H-2K locus. Conversely, DC from B10.MBR/SxEg mice (Ia^k) did not induce nonspecific lytic activity when injected into C57BL/6 (Ia^b) mice despite the fact that they share the K^b element with the recipient mice. However, we found no evidence that the broadly reactive, nonspecific, $CD8^+$ T cells induced by bone marrow-generated DC (17) have a physiologic role in vivo, as judged by the failure of unpulsed or control peptide-pulsed DC to elicit a detectable level of protective immunity in mice inoculated with either E.G7-OVA or EL-4 tumor cells (Fig. 1).

To further evaluate the role of MHC class II and CD^+ T cells in the induction of tumor immunity by DC pulsed with the MHC class I-restricted OVA peptide, mice were depleted of $CD4^+$ or $CD8^+$ T cells, either during immunization with OVA peptide-pulsed DC or during challenge with E.G4-OVA tumor cells. As shown in Figure 4, mice depleted of $CD8^+$ T cells during immunization or during tumor challenge failed to reject the E.G4-OVA tumor. Similarly, mice depleted of $CD4^+$ T cells during immunization failed to mount an antitumor response, but when $CD4^+$ T cells were depleted during challenge, the tumor was rejected. These results, therefore, are consistent with the idea that $CD8^+$ T cells are the primary effector arm in the anti-E.G4-OVA tumor response, and that $CD4^+$ T cells are necessary for the induction of an anti-tumor, $CD8^+$ T cell-dependent response, but do not participate in the effector phase.

Analysis of different antigen pulsing methods of bone marrow-generated DC

The experiment shown in Figures 1*C* and 3 argue that induction of CTL responses and antitumor immunity by bone marrow-generated DC also requires the expression of syngeneic MHC class II Ags on the DC, presumably to present class II-restricted Ags to $CD4^+$ T cells. Thus, while DC pulsed with the H-$2K^b$-restricted OVA peptide were potent inducers of CTL and tumor immunity (Figs. 1–3), the use of protein-pulsed DC may be advantageous, because it would contribute MHC class II-restricted epitopes as well as additional class I epitopes. It was, therefore, of interest to compare several vaccination protocols using OVA peptide or OVA protein to induce CTL and engender antitumor immunity.

It was previously shown that splenic DC pulsed with whole protein in the presence of the lipid DOTAP were able to induce a primary CTL response in vitro (8), and that phagocytic cells exposed to OVA protein in solution could present Ag to an MHC class I-restricted T-T hybridoma (22). We therefore compared three methods of Ag pulsing, using OVA peptide or lipid-OVA protein complex incubated with day 9 DC for 24 h or free OVA protein added to bone marrow cultures on days 4 through 10. For DC pulsing with lipid-OVA protein, we used the method described by Nair et al. (8) with minor modifications that were necessary to eliminate the toxicity of the lipid DOTAP on the bone marrow-generated DC and maximize the uptake of protein (see *Materials and Methods*).

Ag-pulsed DC were injected once into mice and analyzed for induction of the CTL against E.G7-OVA- or OVA peptide-pulsed EL-4 cells as target. As shown in Figure 5, *A* and *B*, DOTAP-OVA protein-pulsed DC induced OVA-specific CTL, but were slightly less effective than OVA peptide-pulsed DC. This small difference between DOTAP-OVA protein and OVA peptide-pulsed DC was observed reproducibly and was also reflected in the level of tumor protection achieved (see below). No OVA-specific CTL were generated when mice were immunized with DOTAP-BSA-pulsed DC (data not shown). Interestingly, incubating day 4 DC cultures with free OVA protein elicited high levels of CTL comparable with those of OVA peptide-pulsed DC. However, additional pulsing with OVA peptide on day 10 did not enhance further CTL induction, suggesting that loading of K^b molecules with OVA peptide was not a limiting factor in either case. The fact that similar levels of CTL were recorded when either E.G7-OVA-pulsed (Fig. 5*B*) or OVA peptide-pulsed EL-4 cells (Fig. 5*D*) were used as targets shows that the bulk of the CTL generated with OVA proteins was directed against the dominant epitope defined by the OVA peptide (amino acids 257–264). Since the effector cells induced by DC pulsed with OVA protein are restricted to the expression of K^b and the specific OVA epitope (Fig. 5, *C* and *D*, and data not shown), it is reasonable to assume that they consist of $CD8^+$ CTL and not $CD4^+$ helper cells.

Free OVA protein was as effective as OVA peptide in generating CTL even when protein was added to day 7 DC cultures (Fig. 6). However, when nonadherent cells from day 9 cultures, highly enriched in mature DC, were pulsed with free OVA protein for 24

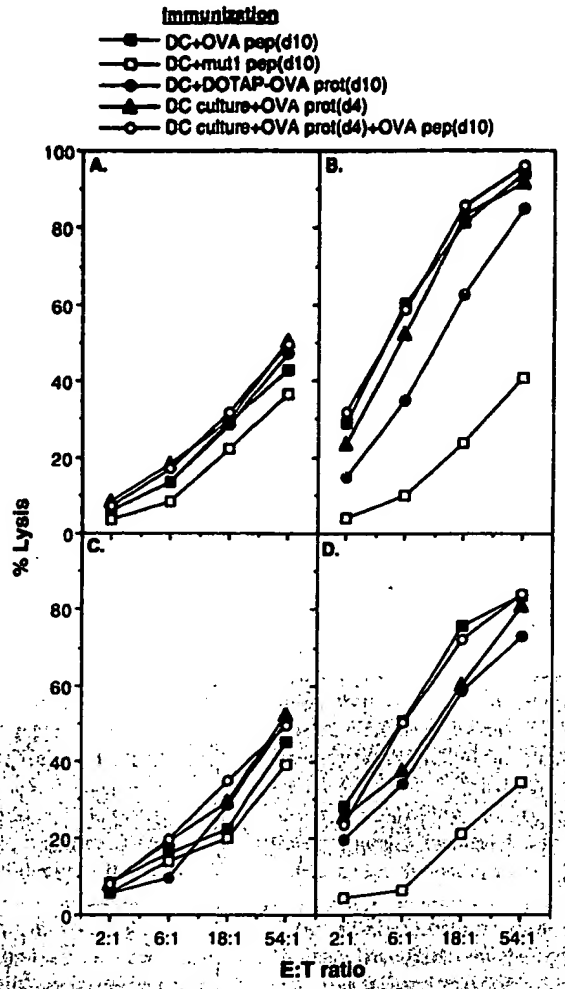

FIGURE 5. Comparison of different Ag pulsing methods on CTL induction. Day 10 DC were pulsed with either OVA peptide or DOTAP-OVA protein complexes as described in *Materials and Methods*, and Ag-pulsed DC (1.5×10^5/mouse) were inoculated i.v. Alternatively, free OVA protein was applied to the DC cultures on day 4, and on day 10, the DC were either injected into mice or first pulsed with OVA peptide and then injected into mice. Seven days later, splenocytes were isolated and restimulated with E.G7-OVA cells. Cytotoxic activity was measured against EL-4 cells (*A*), E.G7-OVA cells (*B*), or EL-4 cells pulsed with 1 mM of mut1 peptide (*C*) or OVA peptide (*D*).

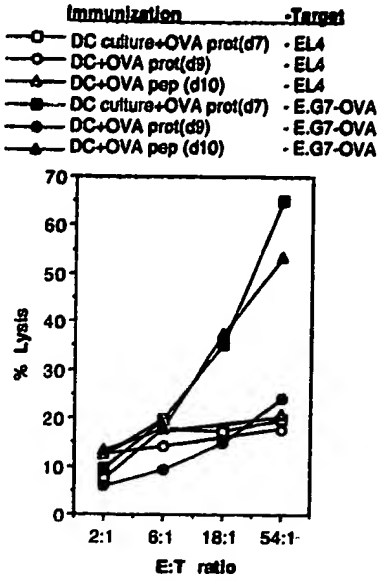

FIGURE 6. CTL induction by DC pulsed with free OVA protein on day 7 or day 9 of culture. OVA protein was added to DC cultures on day 7 and harvested on day 10 for immunization. Alternatively, DC were harvested on day 9 and then incubated for 24 h with the same concentration of the protein. As a positive control, mice were immunized with day 10 DC pulsed with OVA peptide. OVA-protein- or OVA-peptide-pulsed DC were inoculated i.p. Seven days later, nonadherent splenocytes were restimulated with E.G7-OVA cells, and cytotoxic activity was measured against EL-4 and E.G7-OVA cells.

or 6 h, no CTL were generated (Fig. 6 and data not shown, respectively). This observation is consistent with the idea that immature DC or another cell type in the bone marrow cultures are able to process OVA protein and argues that neither fragmentation of the OVA protein preparation nor proteolysis by serum proteases was responsible for generating peptidic epitopes when day 4 or day 7 DC cultures were incubated with OVA protein (Figs. 5 and 6). We cannot, however, rule out the possibility that fragmentation induced by serum proteases became appreciable during a 72-h culture period (day 7 DC), but not during a 24-h culture period (day 9 DC).

We next compared the potency of the antitumor immune response generated by immunizing mice with peptide- or protein-pulsed DC. Mice were immunized once with 1.5×10^5 DC pulsed with OVA peptide, DOTAP-OVA protein complex, free OVA protein, or a combination of OVA peptide and free OVA protein, and challenged with 2×10^7 E.G7-OVA cells. As shown in Figure 7*A*, when tumors were measured 9 days postchallenge, immunization with DC alone or DC pulsed with the mut-1 peptide or DOTAP-BSA did not prevent or delay tumor growth compared with that in mice injected with HBSS alone. On the other hand, no tumor growth was detected in animals immunized with OVA peptide- or OVA protein-pulsed DC, except in one of six animals immunized with DOTAP-OVA protein-pulsed DC. When the tumor size in the control groups reached 3 to 3.5 cm in diameter, 18 to 21 days post-tumor challenge, the animals were killed. Figure 7*B* shows the sizes of tumors in animals immunized with DC pulsed with various forms of OVA Ag 28 days post-tumor challenge. Clearly, under these experimental settings, i.e., when mice where challenged with 2×10^7 E.G7-OVA cells rather than with 1×10^7 cells, none of the immunizations was able to provide complete protection from tumor progression in all animals. It was evident, however, that immunization with DOTAP-OVA protein-pulsed DC was less effective than immunization with peptide-, soluble protein-, or a combination of peptide- and soluble protein-pulsed DC, closely correlating with the CTL data shown in Figure 5 (this experiment was performed twice).

To further compare the potency of OVA peptide-pulsed vs free OVA protein-pulsed DC, mice were immunized with decreasing amounts of Ag-pulsed DC (9×10^4 or 9×10^3 cells) and challenged with 1×10^7 E.G7-OVA tumor cells. As shown in Figure 8*A*, 10 days post-tumor challenge, no tumors were detected in mice immunized with either dose of OVA peptide-pulsed DC, whereas only four of six mice immunized with 9×10^4 OVA protein-pulsed cells and one of six mice immunized with 9×10^3 OVA protein-pulsed DC remained tumor free. On day 25 post-tumor challenge, four of six mice immunized with 9×10^4 OVA peptide-pulsed DC were still tumor free compared with two of six mice immunized with the same dose of cells pulsed with OVA protein.

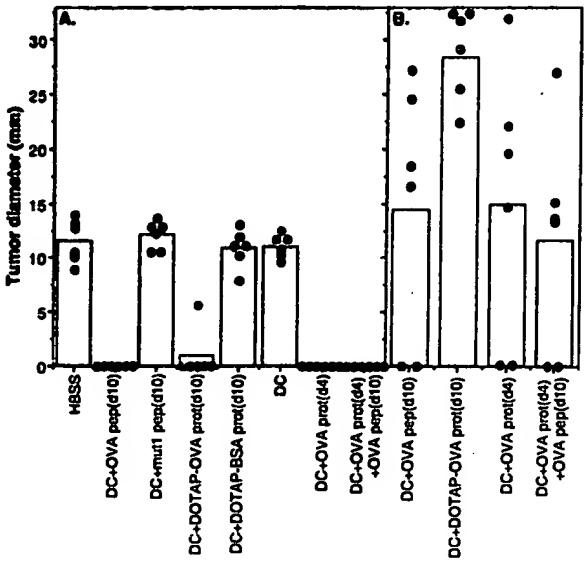

FIGURE 7. Comparison of different Ag pulsing methods on the induction of antitumor immunity. Day 10 DC were pulsed with either OVA peptide or DOTAP-OVA protein complexes as described in *Materials and Methods* and inoculated intravenously (1.5×10^5 cells/mouse). Alternatively, free OVA protein was applied to the DC cultures on day 4, and on day 10, the DC were either injected or first pulsed with OVA peptide and then injected into mice. Mice were challenged intrascapularly with 2×10^7 E.G7-OVA cells 7 days postimmunization. Tumor measurements were taken 9 days (*A*) and 28 days (*B*) post-tumor challenge.

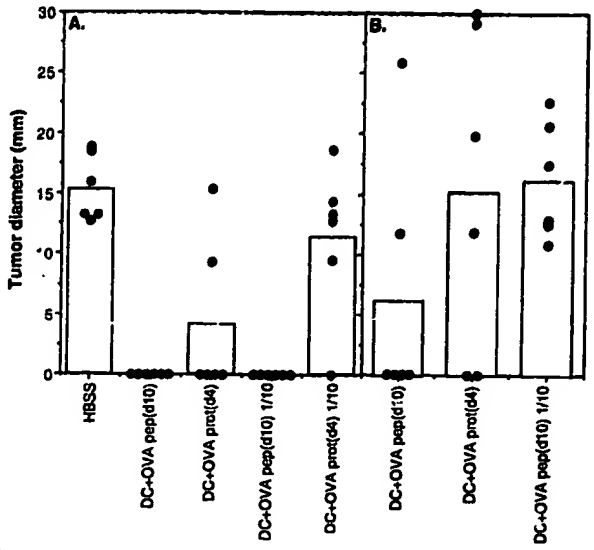

FIGURE 8. Induction of antitumor immunity in mice immunized with bone marrow-generated DC pulsed with OVA peptide or with free OVA protein. Day 10 DC, either cultured with free OVA protein from day 4 or pulsed with OVA-peptide on day 10, were inoculated i.v. Mice immunized with 9×10^4 DC are designated DC+OVA pep or DC+OVA prot, and mice immunized with 9×10^3 DC are designated DC+OVA pep 1/10 or DC+OVA prot 1/10. Mice were challenged intrascapularly with 1.0×10^7 E.G7-OVA cells 7 days postimmunization. Tumor measurements were taken 10 days (*A*) and 25 days (*B*) postchallenge.

The animals in the control group and the group immunized with 9×10^3 OVA protein-pulsed DC were killed before when tumor diameter reached 3–3.5 cm (this experiment was performed once). Thus, it appears that OVA peptide-pulsed DC are more potent inducers of antitumor immunity than OVA protein-pulsed DC.

Discussion

In this study we have shown that bone marrow-generated DC pulsed with OVA Ag induce a protective immune response against a challenge with tumor cells expressing the OVA protein. Several lines of evidence suggest that the DC were highly effective in generating antitumor immune responses. Foremost, immunization with OVA peptide-pulsed DC provided the best protection against tumor challenge, especially when compared with immunization with E.G7-OVA cells or with OVA peptide-loaded RMA-S cells (Fig. 2). As shown in Figure 2, one injection of 3.0×10^5 irradiated E.G7-OVA cells had no measurable antitumor effect, in contrast to one injection of 1.5×10^5 OVA peptide-pulsed DC, which was highly effective. Likewise, immunization with OVA peptide-pulsed RMA-S cells had only a small effect compared with that of DC. This is particularly noteworthy because pulsing of RMA-S cells with peptides generates a high density of MHC epitopes on the cell surface and is highly effective in inducing primary CTL responses in vitro and in vivo (20, 23, 24). Another indication of the remarkable potency of DC is that a single injection of as few as 9×10^3 cells had a measurable antitumor effect (Fig. 8).

In a recent report, Mayordomo et al. have shown that bone marrow-generated DC pulsed with tumor peptides induced protective and therapeutic immunity in the treated animals (25). In that study, high concentrations of GM-CSF and IL-4 were used to generate DC from the bone marrow precursors. The addition of IL-4 served to suppress the generation of macrophages due to the presence of GM-CSF. In our study, DC were generated in the presence of 10- to 50-fold lower concentrations of GM-CSF. Under those conditions, contamination with macrophages was not a factor (17), and the addition of IL-4 to the culture medium had no effect on the expression of a number of cell surface markers, including the B7-1 and B7-2 costimulatory molecules (data not shown).

Efficient induction of MHC class I-restricted CTL responses in vivo was shown to require the participation of Ag-specific MHC class II-restricted $CD4^+$ T cells in some experimental systems (26–29), but not in others (30, 31). In a previous study we have shown that whereas the cytotoxic cells generated in mice immunized with the MHC class I-restricted, OVA peptide-pulsed, bone marrow-generated DC consisted of $CD8^+$ T cells, induction of a CTL response in vivo was dependent on both $CD4^+$ and $CD8^+$ T cells (17). In this study we have shown that in vivo priming of an OVA-specific CTL response and the induction of antitumor immunity require the expression of syngeneic MHC class II molecules on the bone marrow-generated DC presenting the OVA epitope (Figs. 1 and 3 and Table I). These experiments do not rule out the possibility that the MHC-mismatched DC are rejected, thus reducing or eliminating the induction of MHC-restricted lysis by these DC. Yet, in other systems, immunization with cells expressing syngeneic as well as allogeneic MHC increased the induction of MHC-restricted CTL (32). Also, if lysis (nonspecific and OVA specific) induced by DC is reduced or eliminated because of MHC mismatch, then DC from $H2^{bm1}$/By mice injected into C57BL/6 mice would not be expected to elicit nonspecific lysis. Yet, this was not the case (Fig. 3 and Table I). Importantly, the results shown in Figure 3 and Table I should be interpreted in the light of the finding that induction of a $CD8^+$ T cell-mediated antitumor response by bone marrow-derived DC pulsed with the MHC class I-restricted OVA peptide is dependent on $CD4^+$ T cells (Fig. 4). Taken together, these data strongly suggest that in vivo priming of

CTL and induction of antitumor immunity by bone marrow-generated DC also require the presentation of MHC class II-restricted epitopes and activation of $CD4^+$ T cells. This conclusion is supported by the observation of Nonaes et al. that in vitro activation of memory CTL by splenic DC is dependent upon stimulation of a strong $CD4^+$ helper T cell response by the DC (33). A potentially important conclusion from these observations is that vaccination of human patients with peptide-pulsed DC-based vaccines could be further improved by the addition of potent MHC class II epitopes. Moreover, the addition of T helper epitopes would be necessary for peptide-based immunization if human DC were generated in serum-free cultures or in the presence of human serum, as dictated by safety considerations.

MHC class II-dependent priming of CTL and induction of antitumor immunity were seen when mice were immunized with OVA peptide-pulsed DC. Since it is unlikely that this short eight-amino acid peptide encodes a T helper epitope, the likely sources of T helper epitopes are components of the FCS present during generation of DC in the bone marrow culture. This is consistent with the observation that bone marrow-generated DC cultured in FCS-containing medium presented BSA to T cells in vivo, as shown by the fact that T cells from the immunized mice proliferated in vitro in response to BSA (16). To date, we have been unable to generate DC from hemopoietic progenitors in the absence of heterologous components, i.e., in the complete absence of proteinaceous materials or in the presence of mouse serum, which precluded us from a more detailed exploration of this phenomenon using defined MHC class II epitopes.

Ossevoort et al. (34) have shown that splenic DC pulsed with a peptide derived from the human papilloma virus type 16 elicit a potent CTL response and engender protective immunity against a challenge with virus-transformed tumor cells. The splenic DC in this study have been isolated on BSA gradients, which are the likely source of class II epitopes, as suggested by our study and that by Inaba et al. (16). In another report, Mandelboim et al. (35) have shown that RMA-S cells pulsed with a tumor-derived peptide were capable of causing the regression of tumor metastases in tumor-bearing mice. Similarly, we have shown in this and a previous study (17) that a single injection of RMA-S cells pulsed with the OVA peptide into syngeneic mice elicits a CTL response and engenders a significant level of tumor immunity, albeit less than that achieved with peptide-pulsed DC. Since RMA-S cells do not express MHC class II molecules, it appears that induction of CTL and tumor immunity is mediated in this case by presentation of CTL epitopes without the collaboration of $CD4^+$ T cells. A possible reconciliation between these observations and the observation shown in Figure 3 and Table I is that inefficient presentation of CTL epitopes in the absence of class II presentation can be compensated by the presence of a high density of MHC class I-CTL epitope complexes on the surface of the presenting cell.

These observations prompted us to examine the potential benefit of using OVA protein to pulse DC that could serve as a source of both MHC class I and class II epitopes. DC pulsed with free OVA protein were indeed capable of inducing CTL (Fig. 5) and engender antitumor immunity (Figs. 7 and 8); however, they were less efficient than OVA peptide-pulsed DC when only 9×10^3 Ag-pulsed DC were injected into the mice. This difference could stem from the fact that only a subpopulation of DC in the bone marrow culture was capable of redirecting OVA protein into the MHC class I presentation pathway, which became a limiting factor when only 9×10^3 cells were used. The lack of additional benefit seen when mice were immunized with DC pulsed with free OVA protein, especially when combined with OVA peptide pulsing, may reflect the abundance of FCS-derived epitopes presented by the DC, which obscured the potential contribution of epitopes from OVA protein (Fig. 7).

Rock and colleagues have shown that a subset of mouse splenocytes is capable of processing and presenting exogenous OVA Ag in association with MHC class I molecules to an MHC class I-restricted OVA-specific T-T hybridoma (22). Our studies confirm and extend these observations by showing that bone marrow-generated DC present exogenous OVA protein very efficiently, as shown by the fact that mice immunized with a single dose of 1.5×10^5 free OVA protein-pulsed DC elicit a high level of CTL and induce protective antitumor immunity. Interestingly, the data shown in Figure 5, that day 7 DC cultures, but not day 9 mature DC, pulsed with free OVA protein are able to elicit CTL in vivo suggest that the ability of DC to process exogenous OVA protein for MHC class I presentation may be restricted to immature DC precursors or, alternatively, may require the involvement of other cell types present in the bone marrow-derived cultures.

The E.G7-OVA tumor system is a very useful model for evaluating tumor vaccination strategies. It is fast, reproducible, and exhibits a remarkable correlation with in vivo CTL priming data (Ref. 17 and this study), underscoring the importance of the CTL effector arm in antitumor immunity. For example, small differences seen between OVA peptide- and DOTAP-OVA protein-pulsed DC in CTL induction (Fig. 5) are reflected in the level of tumor protection (Fig. 7). The main drawbacks of the E.G7-OVA tumor system are that the tumor rejection Ag expressed in the EL-4 tumor cells, the chicken OVA gene, encodes a strong CTL epitope (19, 36), which is probably uncharacteristic of most tumor Ags. In addition, the potency of the antitumor immune response was assessed by using immunization/challenge protocols rather than by measuring the rejection of preexisting tumors, a more demanding protocol that more closely reflects the situation in the human patient. Nevertheless, this is an excellent system to screen a larger number of immunization conditions for further assessment in more relevant, albeit technically challenging, tumor models, such as were recently used to evaluate genetically modified tumor vaccines (37–40).

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Thank you.

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Functional Comparison of Spleen Dendritic Cells and Dendritic Cells Cultured In Vitro From Bone Marrow Precursors

By Katherine Garrigan, Pisana Moroni-Rawson, Catherine McMurray, Ian Hermans, Nevin Abernethy, Jim Watson, and Franca Ronchese

We have compared dendritic cells (DC) isolated from mouse spleen, or generated in vitro from bone marrow (BM) precursors cultured in granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), for the ability to process and present soluble antigen and stimulate major histocompatibility complex (MHC) Class II-restricted T cells. DC from spleen or BM cultures were equally able to stimulate the in vitro proliferation of allogeneic T cells or of antigen-specific T-cell receptor (TCR)-transgenic T cells. Both DC populations also induced comparable levels of IL-2 secretion by a T-cell hybridoma. Therefore, splenic and BM-derived DC express comparable levels of (Antigen + MHC Class II) ligands and/or costimulatory molecules and have comparable ability to stimulate T-cell responses. When presentation of a native protein antigen, rather than peptide, was evaluated, BM-derived DC were at least 50 times better than splenic DC at stimulating the proliferation of TCR-transgenic T cells. The antigen processing ability of the two populations was similar only when splenic DC were used immediately ex vivo. Therefore, unlike spleen DC, BM-derived DC maintain the capacity to process protein antigen for MHC Class II presentation during in vitro culture. Due to these characteristics, BM-derived DC may represent a useful tool in immunotherapy studies, as they combine high T-cell stimulatory properties with the capacity to process and present native antigen.

DENDRITIC CELLS (DC) are powerful antigen presenting cells (APC) found in skin, airway, and gut mucosa in lymphoid tissue and blood.[1] DC reside in tissues in an immature or nonactivated form characterized by active uptake and processing of antigen,[2] and expression of low levels of T-cell costimulatory molecules. Upon activation, DC are thought to leave tissues and migrate to secondary lymphoid organs where they interact with T lymphocytes and initiate immune responses. Activation is accompanied by the loss of ability to endocytose and process antigen,[2] and by the acquisition of ability to stimulate T lymphocytes through upregulation of T-cell costimulatory molecules.[3] Cognate interaction with T cells further upregulates expression of T-cell costimulatory molecules on DC.[4]

DC show a remarkable capacity to stimulate T-cell responses. Only a few hundred DC are sufficient to stimulate an allogeneic mixed leukocyte reaction (MLR)[5]; in addition, they can prime cytotoxic and T-cell–dependent B-cell responses in vitro.[6,7] Injection of DC in vivo efficiently primes T lymphocytes to proliferation, lymphokine production, and cytotoxic activity.[8-10] Unfortunately, despite the interest caused by these striking characteristics, the use and study of DC have been hampered by difficulties in obtaining them in sufficient numbers and purity. Recently, methods to obtain pure preparations of DC have become available, which involve their in vitro growth and differentiation from blood or bone marrow (BM)-derived precursors.[11-13] The cells obtained with these techniques appear to share many of the properties of spleen-derived DC; however, to what extent this is true is still unclear. To address this question, we have undertaken a comparison of the properties of spleen and BM-derived DC in a number of assays, which are believed to be dependent on distinct properties of the APC. Indeed, spleen and BM-derived DC were similar in most respects, but also showed some remarkable differences.

MATERIALS AND METHODS

Mice. B10.A and BALB/c mice were from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, ME and were maintained at the Animal Facility of the Wellington School of Medicine by (brother × sister) mating. The "AND" mice,[14] transgenic for a T-cell receptor (TCR) specific for pigeon cytochrome *c* (PCC) + I-E^k, were obtained through the courtesy of Dr S. Hedrick, Department of Biology, University of California, San Diego, CA.

In vitro culture media and reagents. All cultures were in Iscove's modified Dulbecco's medium (IMDM) (Sigma Chemical Co, St Louis, MO) containing 2 mmol/L Glutamine (Sigma), 1% penicillin-streptomycin (Sigma), 5×10^{-5} mol/L 2-mercaptoethanol (Sigma) ("complete" IMDM), and 10% fetal bovine serum (Gibco-Life Technologies, Auckland, NZ). PCC was purchased from Sigma; the 88-104 fragment of PCC (KAERADLIAYLKQATAK) was obtained by in vitro chemical synthesis.

Cell lines. The (PCC + I-E^k)-specific hybridoma 2B4[15] was maintained in complete IMDM containing 5% fetal calf serum (FCS). The interleukin (IL)-2–dependent cell line HT-2 was maintained in complete IMDM containing 5% FCS and 100 U/mL human recombinant IL-2 (Roche, Nutley NJ).

Preparation of splenic DC. A modification of the original method of Steinman et al[16] was used. Briefly, spleen cell suspensions were prepared by digestion with a cocktail of 2.5 mg/mL collagenase (Worthington Biochemical Co, Freehold, NJ) and 0.1% DNAse (Sigma) in serum-free medium at 37°C for 2 × 40 minutes; low-density cells were isolated by centrifugation over a 60% Percoll gradient ($\rho = 1.076$). DC were further enriched by differential adherence by incubating cells on 100 mm tissue culture plates (Falcon, Oxnard, CA) in medium containing 5% FCS for 2 hours at 37°C, removing the nonadherent fraction and culturing the remaining cells overnight in medium containing 5% FCS. On the next day, floating cells were collected and analyzed for percentage of DC by fluores-

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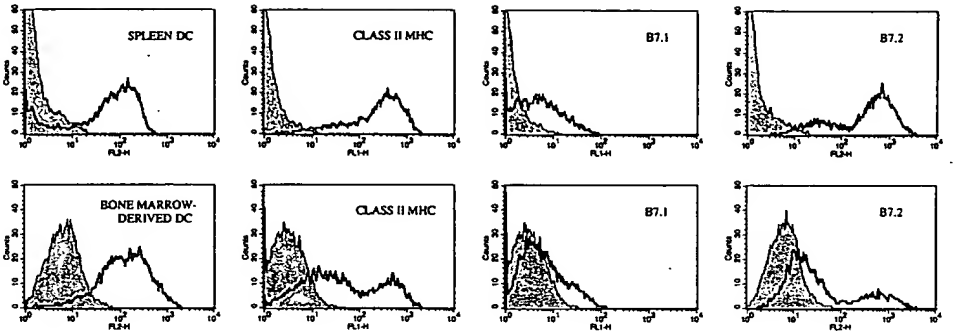

Fig 1. FACS staining of DC preparations from murine spleen (top row), or from BM precursors cultured in vitro (bottom row). Shown are N418 stainings of total populations and MHC Class II, B7.1 and B7.2 stainings of N418+ gated cells. BM cultures set up as described were harvested on day 8.

cent staining and fluorescence-activated cell sorter (FACS) analysis with the DC-specific monoclonal antibody N418.[17]

Growth of BM-derived DC. BM single cell suspensions were cultured in 6-well plates (Falcon) at 2×10^6 cells/5 mL complete IMDM containing 10% FCS, 20 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF), and 20 ng/mL IL-4. Cultures were fed fresh medium and lymphokines every 2 to 3 days, and incubated at 37°C until the time of assay (6 to 8 days).

T-cell stimulation assays. Single cell suspensions were prepared from lymph nodes or thymus as indicated, and depleted of adherent cells by incubation in 100 mm Petri dishes (Falcon) for 1 hour at 37°C in complete IMDM containing 5% FCS. Lymph node suspensions were further depleted of B cells by incubation with Sheep anti-mouse-conjugated Dynabeads (Dynal, Oslo, Norway) for 45′ at 4°C. A total of 2×10^5 AND thymus or lymph node cells, or 5×10^5 BALB/c lymph node cells were plated in 96-well flat bottom microplates (Falcon) together with the indicated number of DC and antigen as indicated, in a total volume of 200 μL. Plates were incubated for 48 or 72 hours as indicated, pulsed with 1 μCi/well TdR-^{3}H (Amersham Int, Little Chalfont, UK), and incubated for a further 5 hours before harvesting. A total of 2B4 cells (10^5/well) were incubated with the indicated numbers of DC and 1 μg/mL PCC peptide in 96-well flat bottom microplates for 24 hours at 37°C. Supernatants were removed and tested for IL-2 content by incubation with the IL-2–dependent cell line HT-2 for 24 hours at 37°C; cell growth was assessed as TdR-^{3}H uptake over 4 hours. All cultures were harvested on a Tomtec 96-well automated cell harvester (Orange, CT) and counted on a Wallac 1450 Microbeta Plus β-counter (Turku, Finland).

FACS analysis. A total of 3 to 10×10^5 cells were stained in 100 μL phosphate-buffered saline (PBS) containing 2% FCS and 0.01% sodium azide in 96-well U bottom microplates (LPI, Milano, Italy) at 4°C for 10′. The monoclonal antibodies N418 and 14.4.4S[18] were affinity purified from tissue culture supernatants and conjugated to biotin or fluorescein isothiocyanate (FITC) as described.[19] Anti-CD80-FITC (B7.1) and anti-CD86-PE (B7.2) monoclonal antibodies were from Pharmingen (San Diego, CA). Streptavidin-phycoerythrin (PE) was from Jackson ImmunoResearch (West Grove, PA). All reagents were used at optimal concentration as experimentally determined. The anti-FcγRII monoclonal antibody (2.4G2)[20] was used at 10 μg/mL to inhibit nonspecific staining. Live cells, identified on the basis of forward scatter (FSC)-side scatter (SSC) profile and propidium iodide dye exclusion, were analyzed on a Becton-Dickinson FACSort using the CellQuest software. Instrument compensation was set in each experiment using single-color stained samples.

RESULTS AND DISCUSSION

FACS analysis of splenic and BM-derived DC. DC in the mouse are identified by the expression of the CD11c marker.[17] We used expression of this marker, as revealed by the N418 monoclonal antibody, to estimate numbers of putative DC in our cell preparations. As shown in Fig 1, CD11c is expressed by a substantial proportion of cells in both spleen and BM-derived cell preparations. In the case of spleen preparations, 60% to 90% of the cells express CD11c; these cells also express high levels of MHC Class II. Most of the cells recovered from BM cultures also expressed the CD11c marker. The number of N418$^+$ cells in the cultures varied with time, increasing rapidly from day 3 to day 6, and remaining approximately constant thereafter (Fig 2). The increase in number of N418$^+$ cells closely paralleled the appearance in culture of nonadherent cells showing characteristic DC morphology. These cells also expressed MHC Class II, and costimulatory molecules, such as B7.1 and B7.2.

T-cell stimulatory function of spleen and BM-derived DC.

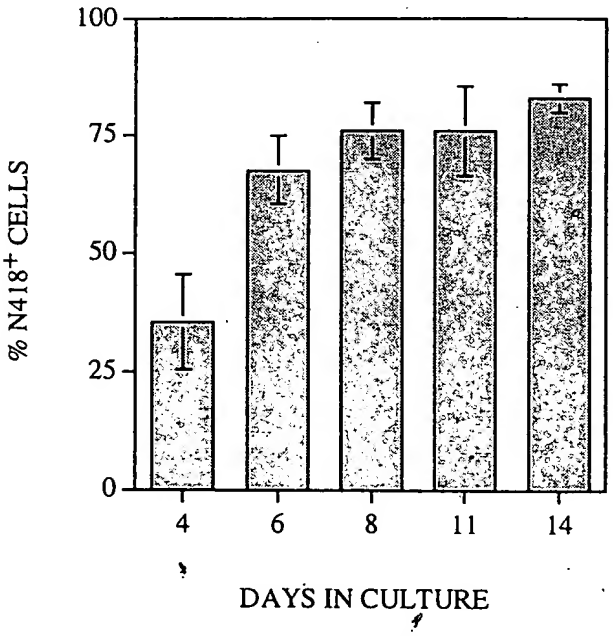

Fig 2. Kinetics of appearance of N418+ cells in BM cultures. Cultures were set up as described in Materials and Methods. Cells were harvested at the indicated times, stained with biotinylated N418 and Streptavidin-PE, and analyzed on a FACSort. Each time point is representative of 2 to 5 experiments.

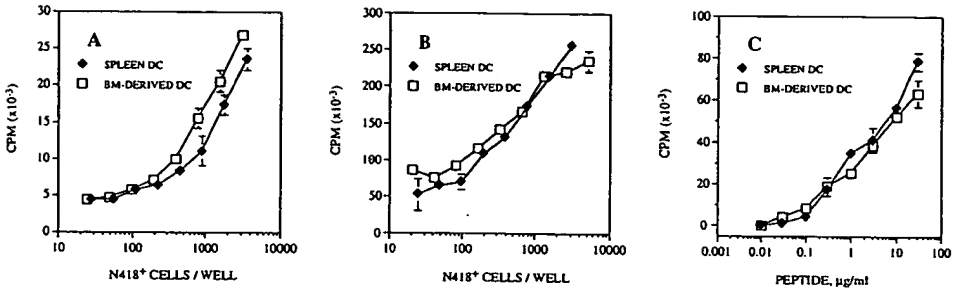

Fig 3. DC isolated from murine spleen, or cultured in vitro from BM precursors, have similar abilities to induce the proliferation of unprimed T cells. Graded numbers of B10.A DC from spleen or BM cultures were incubated for 72 hours with (A) allogeneic BALB/c lymph node T cells or (B) transgenic T cells expressing a (I-E^k + PCC) TCR and PCC peptide (1 µg/mL). (C) Antigen dose-response of transgenic T cells incubated for 48 hours with graded concentrations of PCC peptide antigen and 1,000 spleen or BM-derived DC. The purity of the two populations, as evaluated by N418 expression, was 83% and 94%, respectively.

DC from spleen or from BM cultures were compared for the ability to stimulate T-cell proliferative responses in vitro. Two different assays were used: an allogeneic MLR assay (Fig 3A), where T cells proliferate in response to a whole array of peptide fragments naturally bound to allogeneic MHC Class II, and an antigen specific assay (Fig 3B), where TCR transgenic T cells respond to a specific peptide added in culture. The peptide used in this latter assay, PCC fragment 88-104, does not require processing and is able to directly bind to I-E^k on the surface of APC in the absence of further modification.

DC from either spleen or BM cultures showed comparable ability to induce T-cell proliferation of MHC disparate lymph node populations (Fig 3A) or transgenic T cells (Fig 3B). The observed proliferative responses increased proportionally with the number of DC added in culture, with almost identical dose-response curves for either spleen or BM-derived DC. PCC peptide was used at a suboptimal concentration (1 µg/mL, Fig 3C) to allow a quantitative comparison of the data. The minimum number of DC required to induce a proliferative response ranged between 100 and 500, a number that is consistent with previously reported estimates.[2,5]

The proliferative responses measured in the assays of Fig 3 are essentially proportional to both the number of (antigen + MHC Class II) ligands on the surface of the APC, and the numbers of accessory and costimulatory molecules that are also required to achieve optimal T-cell proliferation. On the basis of the results in Fig 3, we conclude that the overall combination of MHC Class II and costimulatory molecules on spleen and BM-derived DC populations is similar, as shown by the ability of these cells to induce similar T-cell proliferative responses in unprimed T-cell populations.

Antigen processing function of spleen and BM-derived DC. We wished to establish whether spleen and BM-derived DC also had similar abilities to process native protein, and generate (antigen + MHC Class II) ligands for recognition by T cells. To this end, we used an experimental set-up similar to the one used in Fig 3B, with the exception that PCC protein, rather than peptide, was added in culture. As shown in Fig 4A, BM-derived DC were able to take up and process PCC protein and induce T-cell proliferation. Only 2 to 4 times more DC were needed to induce a similar level of T-cell proliferation when whole protein was used, rather than specific peptide. In contrast, splenic DC were very inefficient at inducing T-cell proliferation when PCC protein was used as antigen, being at least 50 times less effective than BM-derived DC. Again, PCC protein was used at a suboptimal concentration to allow a quantitative comparison of the data (Fig 4B).

Ability to process protein antigen appeared to be rapidly lost by spleen DC during culture in vitro. Spleen DC freshly isolated ex vivo and exposed to protein antigen during overnight culture in vitro (see Materials and Methods) displayed a processing ability similar to that of BM-derived DC (Fig 5). Overnight culture of spleen DC in IL-4 was not sufficient to maintain their capacity to process PCC protein (data not

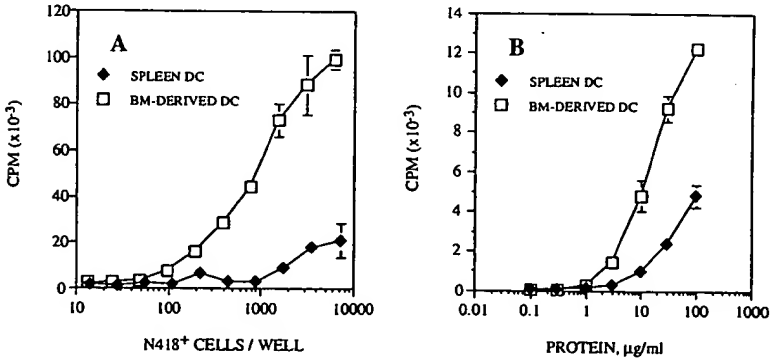

Fig 4. DC cultured in vitro from BM precursors are better than DC isolated from murine spleen at processing antigen. (A) Graded numbers of B10.A DC from spleen or BM cultures were incubated with PCC protein (1 µg/mL) and transgenic T cells expressing a (I-E^k + PCC) TCR for 72 hours. (B) Antigen dose-response of transgenic T cells incubated for 48 hours with graded concentrations of PCC protein and 1,000 spleen or BM-derived DC. The purity of the two populations, as evaluated by N418 expression, was 83% and 94%, respectively.

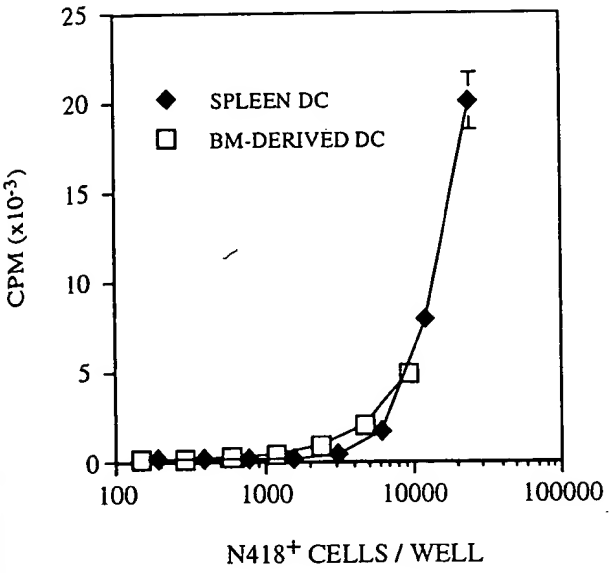

Fig 5. Freshly isolated spleen DC are as efficient as BM-derived DC at processing protein antigen. Low density, adherent spleen cells and bone-marrow-derived DC were pulsed overnight with 1 μg/mL PCC protein; the next day nonadherent cells were collected and incubated with transgenic T cells expressing a (I-E^k + PCC) TCR for 48 hours, always in the presence of 1 μg/mL PCC protein.

shown). Because the same preparations of splenic DC were perfectly able to induce good T-cell proliferation when PCC peptide was used, according to what has been previously reported,[2] we conclude that spleen-derived DC rapidly lose their ability to take up and process native proteins. In contrast, BM-derived DC can efficiently process and present protein antigen even after extended culture in vitro.

Ability of splenic and BM-derived DC to stimulate the T-cell hybridoma 2B4. The assays described in Figs 3 and 4 use as a readout the proliferation of T cells from nonimmunized mice. This response requires that both an antigen-specific and a costimulatory signal are delivered to the T cell from the APC. To better define the contribution of (antigen + MHC Class II) and costimulatory signals to the responses measured, we also compared the APC capacity of splenic and BM-derived DC in a T-cell hybridoma assay. The T-cell hybridoma 2B4 has virtually identical (antigen + MHC Class II) specificity to the TCR transgenic T cells, but, like all hybridomas, it does not require costimulatory signals in order to respond.[21] As shown in Fig 6, when compared on a per cell basis, DC from either spleen or BM cultures induced similar secretion of IL-2 by 2B4 hybridoma cells. This implies that the total amounts of (antigen + MHC) expressed by the two cell types are similar. Indirectly, the data also suggest that the costimulatory signals provided by the two cell types are again similar, as unprimed transgenic T cells (Fig 3B), and a T-cell hybridoma of similar specificity (Fig 6) show identical preferences for APC type.

Conclusions. Methods for the in vitro growth of murine dendritic cells from BM precursors have been described[12,13,22] and are becoming increasingly popular. They offer a number of clear advantages over the traditional methods of purification from lymphoid organs: the procedure is simple, and DC can be reproducibly obtained at high purity and in large numbers from a small number of donors. Due to these reasons, BM-derived DC are quickly becoming the preferred DC for in vivo and in vitro studies. However, it is unclear how closely this in vitro obtained population resembles its in vivo counterpart.

We have undertaken a comparison of the antigen presenting function of DC isolated from murine spleen, or cultured in vitro from BM precursors. We have chosen to concentrate our study on three distinct properties of APC: expression of (antigen + MHC Class II) TCR ligands, capacity to process and present protein antigen, and expression of costimulatory molecules. We have observed that BM-derived DC were as efficient as spleen ones at inducing the proliferation of a peptide-specific TCR-transgenic T-cell population and a polyclonal allogeneic T-cell population. The two DC populations were also equally effective at inducing IL-2 secretion by a peptide-specific T-cell hybridoma. We conclude that BM-derived DC must express high levels of (antigen + MHC Class II) ligands and costimulatory molecules, which are functionally comparable to the levels expressed on spleen DC. Indeed, FACS staining experiments showed that BM-derived DC do express high levels of MHC Class II and costimulatory molecules such as B7.1 and B7.2. This high T-cell stimulatory capacity is perhaps surprising, as it might not have been expected that in vitro–generated population could be functionally as efficient as its ex vivo–derived counterpart. It suggest that BM-derived DC are equivalent to the "mature" DC found in lymphoid organs, which have

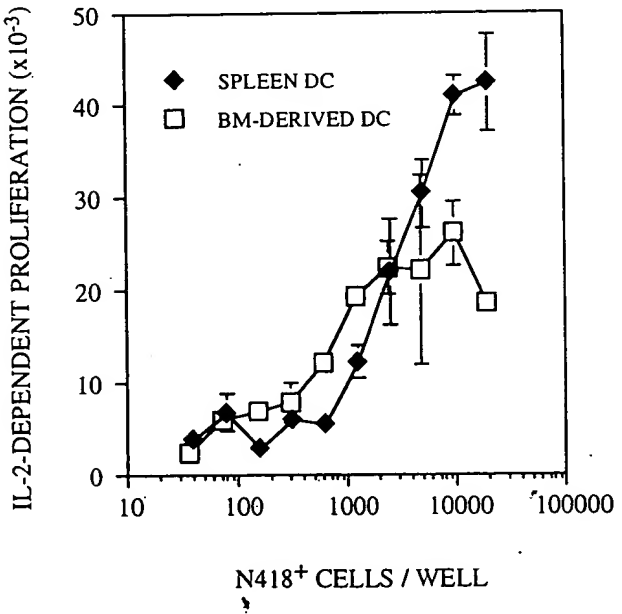

Fig 6. DC isolated from murine spleen, or cultured in vitro from BM precursors, have similar abilities to stimulate IL-2 secretion by the T-cell hybridoma 2B4. Graded numbers of B10.A DC from spleen or BM cultures were incubated with PCC peptide (1 μg/mL) and 2B4 hybridoma cells. Supernatants harvested after 24 hours were tested for IL-2 content by assessing their ability to support the proliferation of the IL-2–dependent cell line HT-2.

upregulated their costimulatory molecules and show good T-cell stimulatory ability.[3,5]

Besides having at least as good a T-cell stimulatory ability, BM-derived DC were also significantly better than spleen DC at processing and presenting a protein antigen. While the antigen processing ability of spleen DC was rapidly lost during the overnight culture required for their isolation, BM-derived DC maintained their antigen processing ability over several days of in vitro culture, suggesting an actual physiological difference between the two cell populations. A good antigen processing ability was recently reported also for human DC cultured from blood precursors[23] and is consistent with the observation that murine BM-derived DC have phagocytic activity and can sensitize mice to bacillus Calmette-Guerin.[24] Such antigen processing ability has so far been considered to be characteristic of the immature forms of DC, the Langerhans cells, which are resident in tissues,[2] and is, therefore, somewhat at odds with the high T-cell stimulatory ability reported above. According to current understanding, DC's antigen processing and costimulatory ability are inversely related. It could then be that the acquisition and maintenance of the stimulatory and antigen processing properties of DC are regulated by cell mediated and soluble factors that are differentially present in culture and in vivo.

In conclusion, DC derived from BM cultures combine the high T-cell stimulatory ability of mature DC with the antigen processing capacity typical of cells, such as dendritic Langerhans cells that reside in the periphery. This unusual combination makes BM-derived DC a potentially very useful tool for experiments requiring the generation of antigen-specific T cells, in vitro and possibly in vivo.

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1. Paglia P, et al. J Exp Med. 1996 Jan 1;183(1):317-22.

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Brief Definitive Report

Murine Dendritic Cells Loaded In Vitro with Soluble Protein Prime Cytotoxic T Lymphocytes against Tumor Antigen In Vivo

By Paola Paglia, Claudia Chiodoni, Monica Rodolfo, and Mario P. Colombo

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Summary

The priming of an immune response against a major histocompatibility complex class I–restricted antigen expressed by nonhematopoietic cells involves the transfer of that antigen to a host bone marrow–derived antigen presenting cell (APC) for presentation to $CD8^+$ T lymphocytes. Dendritic cells (DC), as bone marrow–derived APC, are first candidates for presentation of tumor-associated antigens (TAA). The aim of this study was to see whether DC are able to prime in vivo antigen-specific cytotoxic T lymphocytes after exposure to a soluble protein antigen in vitro. Lacking a well-defined murine TAA, we took advantage of β-galactosidase (β-gal)–transduced tumor cell lines as a model in which β-gal operationally functions as TAA. For in vivo priming both a DC line, transduced or not transduced with the gene coding for murine GM-CSF, and fresh bone marrow–derived DC (bm-DC), loaded in vitro with soluble β-gal, were used. Priming with either granulocyte macrophage colony-stimulating factor–transduced DC line or fresh bm-DC but not with untransduced DC line generated CTL able to lyse β-gal–transfected target cells. Furthermore, GM-CSF was necessary for the DC line to efficiently present soluble β-gal as an $H\text{-}2L^d$–restricted peptide to a β-gal–specific CTL clone. Data also show that a long-lasting immunity against tumor challenge can be induced using β-gal–pulsed bm-DC as vaccine. These results indicate that effector cells can be recruited and activated in vivo by antigen-pulsed DC, providing an efficient immune reaction against tumors.

The cloning of genes encoding tumor-associated antigens (TAA) recognized by T cells in both humans and rodents (1, 2) have reopened the never-ending hope of curing cancer through immunological interventions. Active immunization with such antigens is expected to activate tumor-specific CTL, a process requiring T cell priming in the context of MHC class I pathway (3). CTL induction usually follows antigen processing via endogenous pathway, although exceptions have been reported (4, 5). Host bone marrow–derived APC induced in vivo a CTL-mediated protective immunity against an MHC class I–negative tumor by uptaking and processing for presentation within their own MHC-I (6). Among bone marrow–derived APC, dendritic cells (DC) are the most attractive candidates for this purpose since several studies suggest that they are particularly effective in stimulating both $CD4^+$ and $CD8^+$ naive T lymphocytes (7–12). In fact DC express the costimulatory molecules B7-1 and B7-2, adhesion molecules, and may release soluble factors, all needed for an efficient triggering of the immune response (13). Furthermore, DC were shown to be necessary to generate T cell–mediated tumor-specific immunity in some murine models (14–16). This response was dominated by $CD4^+$ T cells and was generally unable to completely inhibit tumor take unless, as in the case of BCL1 lymphoma, an antiidiotypic antibody response can be generated (16). This limited effect on tumors could be due to the incomplete activation of $CD8^+$ T cells, and particularly of CTL, which are considered as the main effectors able to destroy tumor cells in vivo (3). DC, exposed to soluble antigen in vitro, are able to sensitize antigen-specific T lymphocytes in vivo, in an MHC-II–restricted fashion (7). Since CTLs appear to be necessary to induce tumor rejection in several animal models, we addressed the question of whether DC pulsed in vitro with a soluble antigen were able to induce cytolytic MHC-I–restricted $CD8^+$ T cells as well as a protective antitumor immunity in vivo.

A preliminary account of this work was presented to the Keystone Symposia Conference on Dendritic Cells: Antigen-presenting Cells of T and B Lymphocytes, in Taos, NM, 10–16 March 1995.

Materials and Methods

Animals. Female BALB/c ($H\text{-}2^d$) of 8 and 12 wk of age were purchased from Charles River Laboratories (Calco, Italy). This study was approved by the Institutional Ethic Committee for the use of animals in experimental research.

Cell Lines. D2SC/1 ($H\text{-}2^d$) is a DC line obtained by retroviral immortalization of BALB/c spleen-derived DC, through a previously described procedure (17) and generously provided by Dr. P. Ricciardi-Castagnoli (Consiglio Nazionale delle Ricerche, Milano, Italy). D2SC/1-GM was obtained by transduction of D2SC/1 cell line with the retroviral vector LmGMSN. GM-CSF cDNA was obtained from a murine CTL line by reverse transcription PCR using GM-CSF–specific primer ends modified to include EcoR1 and BamHI sites at 5′ and 3′, respectively. The resulting 517-bp GM-CSF insert was ligated into EcoR1 and BamHI of the LXSN retroviral vector (18), to obtain the LmGMSN vector. Retroviral particles were obtained by *trans*-infection technique, as described (19, 20). D2SC/1-GM cells released ~40 ng/ml per 10^6 cells of GM-CSF as measured by capture ELISA. The clone F1.A11 ($H\text{-}2^d$) expressing β-gal was obtained by transduction of spontaneously transformed BALB/c fibroblast cell line F1 (Colombo, M.P., unpublished results) with the LXSN retroviral vector (19). P13.1 ($H\text{-}2^d$), a P815 mastocytoma line derivative expressing β-gal (5), and the CTL clone 4805B-2 (21) recognizing the β-gal naturally processed, $H\text{-}2L^d$–restricted, epitope p876–884 (22) were kindly provided by Professor H. G. Rammensee (Deutsches Krebsforschungzentrum, Heidelberg, Germany). P815 ($H\text{-}2^d$) and EL4 ($H\text{-}2^b$) tumor lines were used as negative control targets in ^{51}Cr release assay. All cells were maintained in RPMI 1640 supplemented with 10% FCS except for DC lines, which were grown in 5% FCS-supplemented IMDM. The synthetic peptide βGP1 (β-gal, p876–884, TPSPARIGL) was a generous gift of Dr. N. Restifo (National Cancer Institute, Bethesda, MD).

Bone Marrow DC Preparation. Fresh DC were obtained from mouse bone marrow precursors as previously described (23). Bone marrow–derived (bm) DC were used for in vivo priming after 9–11 d of in vitro maturation driven by recombinant mouse GM-CSF at 500 U/ml. The percentage of mature DC was determined by cell surface and intracellular antigens staining and MLR assay as previously described (17). Preparations always resulted in >90% potent MLR stimulator DC (not shown).

In Vivo Immunization and CTL Assay. Before injection in vivo, DC lines as well as fresh bm-DC were exposed in vitro to 100 μg/ml β-gal grade X (Sigma Chemical Co., Milano, Italy) for 18–20 h to allow protein processing. $2\text{–}5 \times 10^6$ D2SC/1 and D2SC/1-GM cells or 5×10^5 fresh DC were injected intraperitoneally in 200 μl PBS after extensive washing in FCS-free medium. After 10–12 d, spleens were removed from three to four immunized mice, pooled, and a single cell suspension prepared by mechanical dissociation. Splenocytes were restimulated at 5×10^6 cells/ml with the synthetic peptide βGP1 (1 μM) or 5×10^5 irradiated (150 Gy) F1.A11 cells/ml in the presence of 20 U/ml recombinant human IL-2 (EUROCETUS, Milano, Italy). After 5–7 d, viable cells were harvested and tested in a ^{51}Cr release assay (24) for their ability to lyse the β-gal–expressing tumor cell line P13.1.

Flow Cytometry. The CD4/CD8 profile of in vivo primed T lymphocytes were analyzed by flow cytometry after conventional mAb staining (17). The following mAbs were used: FITC-conjugated anti-CD4, clone RM4-5, and anti-Thy1.2, clone 53-2.1, and PE-conjugated anti-CD8, clone 53-6.7 (Pharmingen, San Diego, CA). Analysis was performed on a FACScan® (Becton-Dickinson, Milano, Italy); data were collected on 5,000–10,000 viable cells and analyzed using Lysis II® software. To detect cell surface expression of leukocyte markers on DC cell lines, immunostaining was performed as previously described (17) using the following mAbs: M1/42, anti-MHC-I; B21.2, anti-MHC-II ($I\text{-}A^{b,d}$); 2D2C, anti-CD44; M1/69, anti–heat-stable antigen; 3E2, anti-ICAM-1; 1G10, anti-B7-1; GL1, anti-B7-2; and unrelated isotype-matched mAbs as controls.

In Vivo Protection Studies. Mice were immunized following the schedule of Table 1, and control naive animals received in the left rear flank a subcutaneous challenge of F1.A11 living cells (10^4) 10–12 d after vaccination. Mice given the tumor subcutaneously were inspected for tumor growth and size twice a week. The differences in tumor take between control and immunized mice were statistically evaluated by chi-square test, considering a χ^2 value >3.8 as indicative of significant difference at <0.05 level.

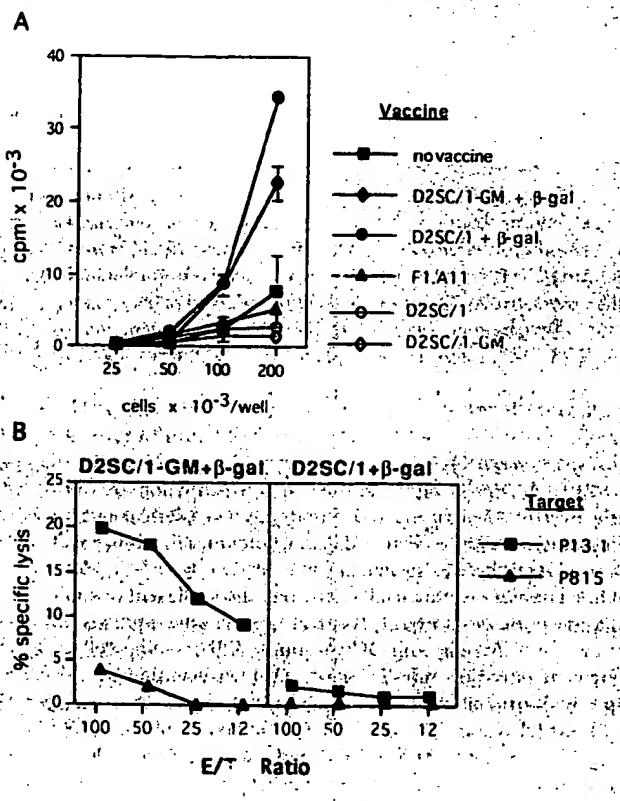

Figure 1. (*A*) Secondary response in mice that have been primed with the antigen-pulsed DC lines. $CD4^+$ T lymphocytes, purified from spleen of naive or from mice immunized with DC lines pulsed in vitro with soluble β-gal or with irradiated F1.A11 tumor cells, were cultured at the indicated cell numbers in presence of β-gal (10 μg/ml) and syngeneic irradiated spleen APC. After 72 h the cultures were pulsed overnight with 1 μCi/well [^{3}H]TdR and harvested on glass fiber filters 18 h later. Incorporated radioactivity was measured by liquid scintillation counting. $CD4^+$ T cells were immunoselected from total spleen cells population by nylon wool purification followed by complement-mediated lysis of $CD8^+$ T cells in presence of the rat anti–mouse mAb 53-6.7 (TIB105), resulting in >85% pure $CD4^+$ T cells as confirmed by immunostaining and flow cytometry (not shown). (*B*) Recognition of MHC-I–restricted β-gal epitope by lymphocytes primed in vivo with DC lines. After immunization with the indicated vaccine (*top*), lymphocytes were restimulated in vitro (5 d) in the presence of βGP1 peptide (1 μM); at the end of the culture lymphocytes were tested in a ^{51}Cr-release assay using P815 (▲) and P13.1 (■) as targets.

Table 1. *Schedule for In Vivo Priming Using bm-DC Pulsed In Vitro with Soluble β-gal*

Group*	Day 0 Vaccine (dose/mouse)	Day 5 Boost (dose/mouse)
1	bm-DC (5×10^5 cells)	—
2	bm-DC (5×10^5 cells)	β-gal (50 μg)
3	bm-DC + β-gal‡ (5×10^5 cells)	—
4	bm-DC + β-gal‡ (5×10^5 cells)	β-gal (50 μg)
5	β-gal (50 μg)	—
6	β-gal (50 μg)	β-gal (50 μg)
7	β-gal (50 μg) in CFA (1:1)	—
8	β-gal (50 μg) in C. parvum (100 μg)	—
9	—	—

*Vaccines and soluble protein boost were administered intraperitoneally. All groups received a challenge of live FLA11 (10^5 cells/mouse; subcutaneously) on day 12 after immunization.
‡Before injection, bm-DC were pulsed in vitro with 100 μg/ml β-gal for 18–20 h.

Results

The DC Line D2SC/1 Exposed to Soluble β-gal In Vitro Primes an Antigen-specific Cytolytic T Cell Response after a Single Immunization In Vivo. To assess the ability of DC to prime a CTL response against a soluble protein in vivo, mice were injected intraperitoneally with $2–5 \times 10^6$ D2SC/1 or D2SC/1-GM cells previously exposed in vitro to soluble β-gal. 10–12 d after immunization, mice were killed and their spleen cells harvested and placed in secondary culture in vitro. As expected, a CD4-mediated β-gal–specific proliferative response was elicited (Fig. 1 *A*). To assess cytolytic activity, splenocytes from vaccinated mice were placed in a secondary in vitro culture with the β-gal synthetic peptide βGP1, or β-gal gene-transduced tumor cells (not shown), and tested, 5 d later, for lytic activity on β-gal–transfected P13.1 and parental P815 cells (Fig. 1 *B*). Splenocytes from mice primed in vivo with D2SC/1 cells + β-gal were unable to specifically lyse P13.1 whereas splenocytes from mice primed with D2SC/1-GM + β-gal lysed P13.1 but not P815. Thus only the priming with the GM-CSF–transduced cell line elicited antigen-specific CTL. Vaccination with DC lines not pulsed with the soluble protein did not induce β-gal–specific CTL, while a proliferative response against FCS components could not be avoided.

GM-CSF Allows the In Vitro MHC-I–restricted Presentation of Soluble Antigen by D2SC/1 Cell Line. Further investigation of the effect of GM-CSF transduction on the DC line D2SC/1 did not reveal modifications in the pattern of cell surface markers but, as already observed after addition of recombinant mouse GM-CSF to another immortalized cell line (17), GM-CSF–transduced DC became free in suspension and acquired a more dendritic morphology (not shown).

Moreover, that GM-CSF modifies the ability of D2SC/1 to present β-gal was clearly shown by experiments in which D2SC/1 and D2SC/1-GM pulsed with either βGP1 peptide or soluble β-gal were used as targets of the β-gal–specific CTL clone 0805.B. In fact, while both D2SC/1 and D2SC/1-GM were lysed if pulsed with the peptide βGP1 (Fig. 2 *A*), only D2SC/1-GM was lysed after pulsing with soluble β-gal (Fig. 2 *B*). This result clearly indicates that the peptide was correctly displayed within the

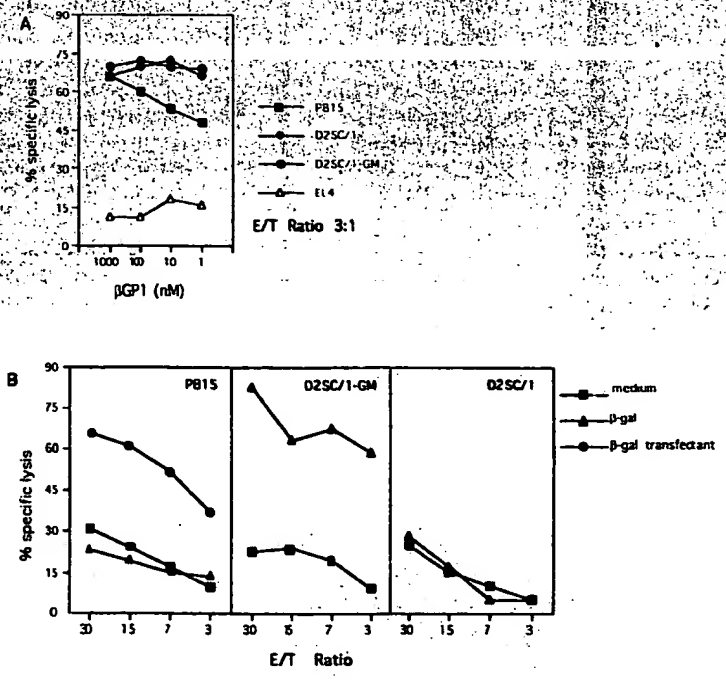

Figure 2. Recognition of β-gal naturally processed epitope by β-gal–specific 0805.B CTL clone. (*A*) Recognition of βGP1-pulsed targets; (*B*) recognition of soluble β-gal–pulsed targets. Lytic activity was assayed in a ^{51}Cr release after incubation of target cells with soluble β-gal (100 μg/ml, 18–20 h) or βGP1 peptide (1–0.001 μM; 2 h).

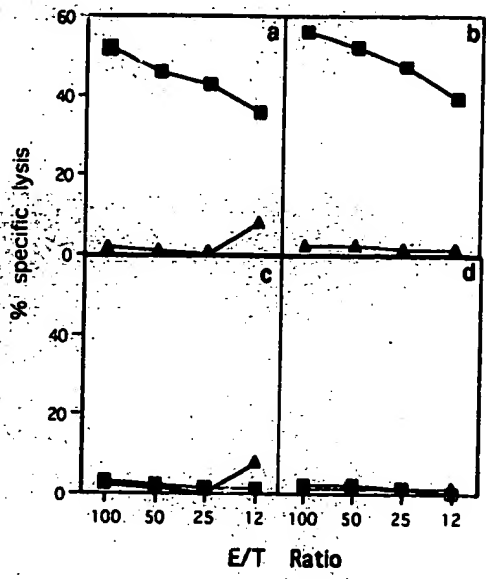

Figure 3. Recognition of MHC-I–restricted β-gal epitope by lymphocytes primed in vivo with bm-DC. After immunization with the indicated vaccine, lymphocytes were restimulated in vitro (5 d) in the presence of βGP1 peptide. At the end of the culture, live lymphocytes were tested in a ^{51}Cr release assay using P815 (▲) and P13.1 (■) as targets. (*a*) bm-DC pulsed with β-gal; (*b*) bm-DC pulsed with β-gal plus β-gal boost; (*c*) β-gal; (*d*) β-gal plus β-gal boost.

MHC-I of both DC lines but that only DC transduced with GM-CSF can process the soluble protein for MHC-I presentation.

Fresh bm-DC Process Soluble β-gal In Vitro and Prime Antigen-specific CTL In Vivo. To investigate whether the in vivo priming of CTL is a property restricted to immortalized DC lines, experiments were run in parallel by using bm-DC. Fresh DC were exposed to soluble antigen in vitro with the same procedure and concentration used for D2SC/1 cells and injected intraperitoneally in BALB/c mice (Table 1). 500,000 bm-DC pulsed in vitro with soluble β-gal were sufficient to prime antigen specific CTL in vivo, whereas no evidence of CTL activation was seen in mice immunized once or twice with soluble β-gal or with unloaded bm-DC (Fig. 3).

Lymphocytes primed in vivo with DC and expanded by secondary in vitro cultures, were enriched in T cells. Particularly, we found that $CD8^+$ cells were 55–70% and 15–35% in DC versus tumor cell–primed lymphocytes, respectively. Furthermore, lymphocytes from mice primed with DC showed a blastlike morphology, indicating a probable entry in G1 phase, since their forward scatter/side scatter parameters were 500:250 while parameters of lymphocytes from mice primed with tumor cells were 200:50, the latter without a significant difference from naive lymphocyte size (Table 2).

Vaccination with DC Pulsed with β-gal Protects Mice Against a Challenge with β-gal–transduced Tumor Cells. To test whether the immune response induced by bm-DC can induce resistance against a challenge with live tumor cells, 10–12 d after immunization mice were injected subcutaneously with 10^4 viable F1.A11 cells. Immunization with bm-DC pulsed with soluble β-gal protected 60% of challenged mice while mice receiving also a soluble β-gal boost 3 d after the priming with pulsed bm-DC were completely protected against tumor challenge. Mice immunized with β-gal, boosted or not with the soluble protein, did not show statistically significant difference in tumor take as compared to naive mice which received the same challenging dose. No protection was seen also in mice receiving unloaded bm-DC accompanied or not by a boost of soluble protein (Fig. 4 *A*). Vaccination with soluble β-gal admixed with CFA or *Corynebacterium parvum* adjuvants did not result in tumor protection (Fig. 4 *B*).

Discussion

To directly investigate the in vivo priming of a CD8-mediated T cell response after prophylactic vaccination with soluble protein loaded DC, we took advantage of a previously described immortalized DC line (17) to set up the model, and from the complete set of reagents that an antigen like β-gal may offer. In fact, β-gal–soluble protein, its immunogenic peptide as well as a retroviral vector able to transduce the gene into tumor cells, were all available. In addition, β-gal has been chosen because, as soluble protein,

Table 2. *Activation of In Vivo Primed Lymphocytes after In Vitro Secondary Culture with β-gal–derived Synthetic Peptide*

Vaccine	Mean forward scatter	Percent CD4	Percent CD8	CD4/CD8
bm-DC	200	24	11	2.18
bm-DC + β-gal boost	200	32	9	3.5
bm-DC + β-gal*	500	6	70	0.08
bm-DC + β-gal + β-gal boost	400	20	60	0.33
β-gal	200	65	22	3.03
β-gal + β-gal boost	200	70	24	2.93
F1.A11	200	48	38	1.26

Lymphocytes were restimulated in presence of βGP1 (1 μM) for 5 d in vitro.
*Before injection, bm-DC were pulsed in vitro with 100 μg/ml β-gal for 18–20 h.

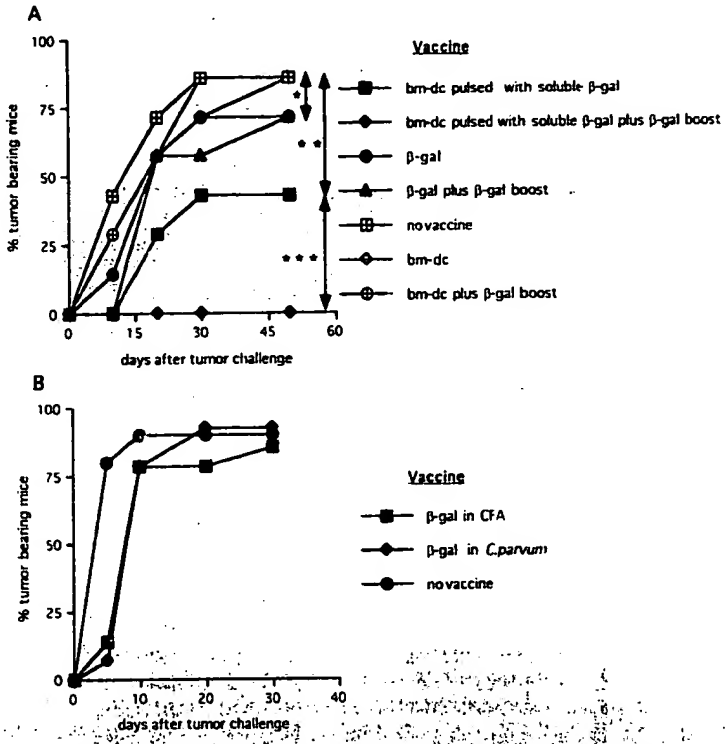

Figure 4. Ability of immunized mice to reject a β-gal–expressing tumor challenge. After immunization with the indicated vaccine, BALB/c mice were injected subcutaneously with 10^4 F1.A11 tumor cells. Tumor growth was inspected twice a week by palpation. Seven mice were included in each group. *Statistically not different; **$P < 0.05$; ***$P \leq 0.01$.

it lacks intrinsic properties that allow its entry into the cell outside the endosomal compartment and because it is unable to stimulate CTL in vivo (5).

We observed that DC lines loaded in vitro with soluble antigen were able to activate antigen-specific CTL if transduced with GM-CSF gene. This confirms previous observations which indicate GM-CSF as a "pro-presentation" cytokine (25). In fact, GM-CSF is able to enhance the immune response initiated by DC, including immunity against tumors, by directly stimulating maturation of their accessory properties (15, 26). Availability of DC lines either transduced or not with the GM-CSF gene was instrumental to define clearly the role of GM-CSF, since bm-DC could be obtained only in the presence of GM-CSF, thus lacking the control counterpart. Our results demonstrate that GM-CSF can activate a pathway of antigen processing that allows exogenous soluble protein to be presented by MHC-I molecules as shown by the recognition and killing of GM-CSF–transduced DC loaded with soluble β-gal by a CTL clone specific for the β-gal, H-$2L^d$–restricted epitope. Furthermore, CTL activation in vivo was obtained by vaccination with fresh bm-DC loaded with soluble β-gal in vitro, and vaccinated mice were completely protected against live tumor challenge if boosted with the soluble antigen. Here, GM-CSF was necessary to drive maturation of DC in vitro, while antigen boost shows that concomitant recall of DC-primed T helper cells is likely to be beneficial for sustaining a protective immune response against tumors as it was shown to occur for the induction of reaction against influenza virus in vitro (27). Along with CTL, Th were certainly activated by immunization with β-gal–loaded DC since both antigen-specific $CD4^+$ T_c cell proliferation and anti–β-gal antibody production were observed (not shown).

That β-gal could be considered a model to overcome the limited availability of well-characterized tumor antigen in the mouse is supported by our preliminary results showing that bm-DC pulsed with a 20^{mer} length peptide encompassing the Arg^{12} mutation of the K-*ras* oncogene which, by itself, can not be accommodated within the MHC-I groove, immunize naive mice against a fibrosarcoma carrying this mutation (Carbone, G., et al., manuscript in preparation). Identification and cloning of tumor antigens expressed by melanomas (2) and identification of immunogenic peptides within mutated or overexpressed oncogenes, i.e., RAS and HER-2/neu (3), prompted the possibility to immunize cancer patients with well-defined T cell epitopes. Since immunogenicity of antigenic peptides may depend on the type of adjuvant, pulsed DC may represent the ideal cell-based vaccine even for human tumors, especially now that obtaining a consistent number of DC from human $CD34^+$ precursor by CSF administration is possible (28).

We are particularly grateful to Professor Ralph Steinman for critical reading of the manuscript and suggestions. We thank Dr. G. Parmiani for valuable discussion, Dr. P. Ricciardi-Castagnoli for providing the D2SC/1 cell line, and Mr. I. Arioli for his expertise in animal handling.

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Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo.

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Paglia P, Chiodoni C, Rodolfo M, Colombo MP.

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The priming of an immune response against a major histocompatibility complex class I-restricted antigen expressed by nonhematopoietic cells involves the transfer of that antigen to a host bone marrow-derived antigen presenting cell (APC) for presentation to CD8+ T lymphocytes. Dendritic cells (DC), as bone marrow-derived APC, are first candidates for presentation of tumor-associated antigens (TAA). The aim of this study was to see whether DC are able to prime in vivo antigen-specific cytotoxic T lymphocytes after exposure to a soluble protein antigen in vitro. Lacking a well-defined murine TAA, we took advantage of beta-galactosidase (beta-gal)-transduced tumor cell lines as a model in which beta-gal operationally functions as TAA. For in vivo priming both a DC line, transduced or not transduced with the gene coding for murine GM-CSF, and fresh bone marrow-derived DC (bm-DC), loaded in vitro with soluble beta-gal, were used. Priming with either granulocyte macrophage colony-stimulating factor-transduced DC line or fresh bm-DC but not with untransduced DC line generated CTL able to lyse beta-gal-transfected target cells. Furthermore, GM-CSF was necessary for the DC line to efficiently present soluble beta-gal as an H-2Ld-restricted peptide to a beta-gal-specific CTL clone. Data also show that a long-lasting immunity against tumor challenge can be induced using beta-gal-pulsed bm-DC as vaccine. These results indicate that effector cells can be recruited and activated in vivo by antigen-pulsed DC, providing an efficient immune reaction against tumors.

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Immunotherapeutic potential of tumor antigen-pulsed and unpulsed dendritic cells generated from murine bone marrow.

Yang S, Darrow TL, Vervaert CE, Seigler HF.

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Dendritic cells (DC) are highly efficient antigen-presenting cells able to capture, process, and present antigens to naive and primed T-cells. In this study, we have investigated the ability of DC, derived from murine bone marrow and pulsed with tumor cell extracts, to induce regression of preexisting tumors. In an experimental model of B16 melanoma in B6 mice, a significant reduction in metastatic nodules in the lungs was observed in tumor-bearing animals treated with either DC alone or DC pulsed with tumor extracts. Kinetic studies demonstrate that the efficacy of these tumor vaccines is inversely related to tumor burden. In this model, tumor-specific cytotoxic T-cells (CTL) could also be induced in vitro from spleen cells derived from tumor-bearing animals treated with DC pulsed with tumor extracts. Untreated mice had no CTL. Furthermore, DC alone elicited tumor-specific CTL responses in tumor-bearing mice, but not in naive mice. Immune cell depletion experiments show that the therapeutic effects of DC are primarily mediated by CD8+ T-cells, while CD4+ T-cells and NK cells are involved in DC-mediated antitumor immunity to a limited extent. These results illustrate the potential use of DC and DC pulsed with tumor extracts as potent therapeutic reagents for cancer and provide a rationale for using DC in vivo to eliminate disseminated tumors or residual tumor deposits following surgery.

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Induction of antitumor immunity using bone marrow-generated dendritic cells.

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We have previously shown that bone marrow-generated dendritic cells (DC) pulsed with a class I-restricted peptide are potent inducers of CD8+ CTL. In the present study we have investigated whether bone marrow-generated DC are capable of inducing antitumor immunity. We show that a single immunization with DC pulsed with OVA peptide was highly effective in eliciting a protective immune response against a challenge with tumor cells expressing the OVA gene (E.G7-OVA), more so than immunization with irradiated E.G7-OVA cells, OVA peptide-pulsed RMA-S cells, or free OVA peptide mixed with adjuvant. The addition of free OVA protein to day 4 or day 7 bone marrow cultures, but not to day 9 mature DC, was also effective in eliciting CTL and engendering antitumor immunity, but was less effective than peptide-pulsed DC. Induction of CTL and antitumor immunity by bone marrow-generated DC pulsed with the class I-restricted OVA peptide correlated with the expression of syngeneic MHC class II molecules on the DC. This and the fact that induction of tumor immunity was dependent on CD4+ T cells suggest that in vivo priming of CTL and induction of antitumor immunity by bone marrow-generated DC also require the presentation of MHC class II-restricted epitopes and activation of CD4+ T cells. This observation has potentially important implications to the use of peptide-pulsed DC in clinical immunotherapy.

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Functional comparison of spleen dendritic cells and dendritic cells cultured in vitro from bone marrow precursors.

Garrigan K, Moroni-Rawson P, McMurray C, Hermans I, Abernethy N, Watson J, Ronchese F.

Malaghan Institute of Medical Research, Wellington South, New Zealand.

We have compared dendritic cells (DC) isolated from mouse spleen, or generated in vitro from bone marrow (BM) precursors cultured in granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), for the ability to process and present soluble antigen and stimulate major histocompatibility complex (MHC) Class II-restricted T cells. DC from spleen or BM cultures were equally able to stimulate the in vitro proliferation of allogeneic T cells or of antigen-specific T-cell receptor (TCR)-transgenic T cells. Both DC populations also induced comparable levels of IL-2 secretion by a T-cell hybridoma. Therefore, splenic and BM-derived DC express comparable levels of (Antigen + MHC Class II) ligands and/or costimulatory molecules and have comparable ability to stimulate T-cell responses. When presentation of a native protein antigen, rather than peptide, was evaluated, BM-derived DC were at least 50 times better than splenic DC at stimulating the proliferation of TCR-transgenic T cells. The antigen processing ability of the two populations was similar only when splenic DC were used immediately ex vivo. Therefore, unlike spleen DC, BM-derived DC maintain the capacity to process protein antigen for MHC Class II presentation during in vitro culture. Due to these characteristics, BM-derived DC may represent a useful tool in immunotherapy studies, as they combine high T-cell stimulatory properties with the capacity to process and present native antigen.

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Antigen processing: cultured lymph-borne dendritic cells can process and present native protein antigens.

Liu LM, MacPherson GG.

Sir William Dunn School of Pathology, University of Oxford, UK.

Langerhans' cells (LC) cultured for 1-3 days lose their ability to process native protein antigens but acquire the ability to stimulate resting T cells as assessed in an allogeneic mixed lymphocyte response (MLR). Lymph-borne dendritic cells (L-DC) are physiologically involved in the transport of antigens to lymph nodes but it is not known whether these cells lose the ability to process antigens in culture. To investigate this, we cultured L-DC derived from the intestine for 20-72 hr and tested their ability to process and present antigens. Our results show that these L-DC are able to present antigen to primed spleen T cells as effectively as fresh cells. To exclude the possibility that commercial ovalbumin (OVA) preparations contain peptides which might bind directly to major histocompatibility complex (MHC) molecules, OVA was filtered through Sephadex G50 and the peak fractions used as antigen. The results show that cultured L-DC are also able to present G50-filtered OVA efficiently to primed spleen T cells. More importantly, these G50-OVA-pulsed L-DC are able to prime naive T cells specifically in vivo. Chloroquine inhibited the ability of both fresh and cultured L-DC to present antigen to primed T cells but did not inhibit their ability to stimulate a MLR, indicating that processing was a necessary step for antigen presentation. Taken together, these results clearly show that cultured L-DC are active in processing and presenting native antigens and the hypothesis proposed for LC does not apply to rat lymph-borne dendritic cells. The physiological significance of these observations is discussed.

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Antigen-presenting cell function of dendritic cells and macrophages in proliferative T cell responses to soluble and particulate antigens.

Kapsenberg ML, Teunissen MB, Stiekema FE, Keizer HG.

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The capacity of dendritic cells (DC) and macrophages (M phi) to present soluble and particulate antigen was tested in an ovalbumin (OVA)-specific T cell proliferation assay. In a comparative investigation we found that both DC and M phi were able to present soluble OVA, but that only M phi could present insolubilized OVA to T cells. DC were found to be able to present OVA in collaboration with M phi. The failure of DC to present insolubilized OVA is probably caused by their inability to endocytose these antigens. DC appeared not to endocytose substantial amounts of soluble OVA either. In contrast to M phi, antigen presentation by DC is not blocked by lysosomotropic drugs. Taken together, these observations suggest that DC can present soluble protein antigens without intracellular degradation.

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